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PEPTIDE-BASED METHOD FOR MONITORING GENE EXPRESSION IN A HOST CELL

The present invention relates a method for monitoring the expression level of a gene in a host cell by modulating the activity of a regulatory biomolecule, comprising the steps of: (a) transforming a cell expressing a regulatory biomolecule with a nucleic acid molecule comprising an open reading frame encoding an interaction partner of said biomolecule in expressible form, wherein (i) said regulatory biomolecule is either a nucleic acid binding molecule that effects its regulatory activity upon binding or an allosterically controlled ribonucleic acid molecule; and (ii) the interaction partner of the biomolecule is encoded by a nucleic acid molecule comprising: (1) a nucleic acid sequence encoding a tagged (poly)peptide, (2) a nucleic acid sequence encoding a tagged (poly)peptide or a peptide tag, a selectable marker gene and additional nucleotide sequences for site specific, in-frame integration of said nucleic acid molecule into the coding sequence of at least one host (poly)peptide of interest, wherein said tag comprises the interacting residues of the interaction partner, or (3) a nucleic acid sequence encoding a peptide tag, a selectable marker gene and additional nucleotide sequences for transposase-mediated random integration of said nucleic acid molecule into the coding sequence of at least one host (poly)peptide of interest, wherein said tag comprises the interacting residues of the interaction partner and (b) assessing the expression level of the gene. Furthermore, the present invention relates to a method of producing and/or selecting a compound capable of modulating the activity of a nucleic acid binding protein comprising the steps of: (a) conducting a selection of compounds with the nucleic acid binding target protein under conditions allowing an interaction of the compound and the nucleic acid binding protein; (b) removing unspecifically bound compounds; (c) detecting specific binding of compounds to the nucleic acid binding target protein; (d) expressing in a cell, the nucleic acid binding protein and providing in trans the coding sequence of at least one indicator gene, wherein said coding sequence is under control of the target sequence of the nucleic acid binding protein; (e) adding a candidate compound to the cell of step (d); (f) determining the amount or activity of

5 the indicator protein, wherein a reduced or increased amount of indicator protein is indicative of compounds, capable of modulating the activity of the nucleic acid binding protein; (g) selecting compounds capable of modulating the activity of the nucleic acid binding protein. Moreover, the present invention relates to nucleic acid molecules, polypeptides, expression vectors, transposable genetic elements, host
10 cells, ensembles of host cells and a non-human animal comprising said host cells. Finally, the present invention relates to a kit comprising the nucleic acid molecule, the (poly)peptide, the vector, the host cell or the ensemble of host cells of the present invention in one or more containers.

Several documents are cited throughout the text of this specification. The disclosure
15 content of the documents cited herein (including any manufacturer's specifications, instructions, etc.) is herewith incorporated by reference.

Despite tremendous advances in our comprehension of the molecular basis of infectious diseases or diseases such as cancer, substantial gaps remain both in our understanding of disease pathogenesis and in the development of effective
20 strategies for early diagnosis and for treatment. Proteomic approaches to investigate disease will overcome some of the limitations of other approaches. The opportunities as well as the challenges facing disease proteomics are formidable. Particularly promising areas of research include: delineation of altered protein expression, not only at the whole-cell or tissue levels, but also in subcellular
25 structures, in protein complexes and in biological fluids; the development of novel biomarkers for diagnosis and early detection of disease; and the identification of new targets for therapeutics and the potential for accelerating drug development through more effective strategies to evaluate therapeutic effect and toxicity.

Despite earlier predictions to the contrary, infectious diseases remain a leading
30 cause of worldwide death. A complicating factor in therapy for infectious diseases is the development of resistance to commonly used drugs (for example, as has occurred in tuberculosis), which heightens the need for developing effective new therapies. Interest in the application of proteomics to microbiology goes back at least two decades, with the pioneering work of Fred Neidhardt to characterize
35 protein expression patterns in *Escherichia coli* under different growth conditions (VanBogelen, R. A., Schiller, E. E., Thomas, R. D. & Neidhardt, F. C. Diagnosis of

5 cellular states of microbial organisms using proteomics. Electrophoresis 20, 2149-2159 (1999)).

The complete sequencing of a number of microbial genomes has provided a framework for characterizing the role of various proteins and their relevance for pathogenic persistence or for disease progression. A crucial aspect of the
 10 continuing fight against pathogenic organisms is the question of whether or not it will be possible to identify those proteins within the pathogenic proteome, the expression of which is essential for the survival of the pathogen. Many of these proteins are likely to be tissue specific proteins because an optimal adaptation to the local conditions within the infected host is probably the most important prerequisite
 15 for survival of the pathogen. Due to their outstanding relevance for survival, these tissue-specific proteins are likely to be prime targets for drug development.

At present, the characterization of tissue or environment specific gene expression is primarily based on biochemical analysis of individual proteins or protein sets [see (Hanash, 2003) and (Jain, 2000) for reviews and references cited therein]. For
 20 proteome analysis, pathogenic microorganisms are often grown *in vitro* (in a chemostat, for example) under conditions known or assumed to mimic conditions in a host organism [*Streptococcus mutans* - (Len et al., 2003); *Salmonella typhimurium* - (Deiwick et al., 2002); *Pseudomonas aeruginosa* - (Guina et al., 2003); *Leishmania infantum* - (El Fakhry et al., 2002); *Mycobacterium tuberculosis* - (Rosenkrands et
 25 al., 2002)]. The quest for identifying new tissue- or disease-specific proteins is hampered by the fact that methods like tissue micro-dissection, Protein Chip Array technologies, MALDI-TOF mass spectrometry or mass spectrometric analysis of *in situ* tissue sections are time-consuming, expensive and technically demanding (Hanash, 2003; von Eggeling et al., 2000); they are also often limited by the
 30 presence of buffer components in biological samples, the heterogeneity of the protein sample or the lack of adequate amounts of sample for analysis. Tagging of all identified ORFs allows either separation of cells expressing a tagged protein by FACS from those not expressing a tagged protein and quantification in the case of highly-expressed or strongly-localized proteins in the case of a GFP-tag (Huh et al.,
 35 2003) or a precise quantification of a tagged protein's expression level in the case of a TAP-tag (Ghaemmaghami et al., 2003). Thus, quantification and visual detection

5 still requires the introduction of two independent tags per protein. While the *in vitro* manipulation of growth conditions allows the isolation of large amounts of protein samples, this must not faithfully represent the situation in a living host organism.

Thus, the technical problem underlying the present invention was to provide means and methods for identifying gene expression under specific growth conditions
10 including tissue specific gene expression in a host cell or in an *in vivo* animal model for a disease. The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a method for monitoring the expression level of a gene in a host cell by modulating the activity of a regulatory biomolecule,
15 comprising the steps of: (a) transforming a cell expressing a regulatory biomolecule with a nucleic acid molecule comprising an open reading frame encoding an interaction partner of said biomolecule in expressible form, wherein (i) said regulatory biomolecule is either a nucleic acid binding molecule that effects its regulatory activity upon binding or an allosterically controlled ribonucleic acid
20 molecule; and (ii) the interaction partner of the biomolecule is encoded by a nucleic acid molecule comprising: (1) a nucleic acid sequence encoding a tagged (poly)peptide, (2) a nucleic acid sequence encoding a tagged (poly)peptide or a peptide tag, a selectable marker gene and additional nucleotide sequences for site specific, in-frame integration of said nucleic acid molecule into the coding sequence
25 of at least one host (poly)peptide of interest, wherein said tag comprises the interacting residues of the interaction partner, or (3) a nucleic acid sequence (corresponding to a transposable genetic element) encoding a peptide tag, a selectable marker gene and additional nucleotide sequences for transposase-mediated random integration of said nucleic acid molecule into the coding sequence
30 of at least one host (poly)peptide of interest, wherein said tag comprises the interacting residues of the interaction partner and (b) assessing the expression level of the, *thus, tagged* gene.

The term "(poly)peptide" refers alternatively to peptide or to polypeptide. Peptides conventionally are covalently linked amino acids of up to 30 residues, whereas
35 polypeptides (also referred to as "proteins") comprise 31 and more amino acid residues.

5 The term "biomolecule" relates to a (poly)peptide or an RNA molecule or a ribonucleoprotein which is capable of modulating the amount of gene expression, preferably the amount of protein expression. "Modulating gene expression" means inhibiting or enhancing gene expression. An example of a biomolecule which is capable of inhibiting the amount of gene expression is the Lac repressor. By binding

10 to its recognition site, the Lac repressor is capable of inhibiting the expression of open reading frames which are located downstream of this recognition site. Alternatively, the biomolecule can be an enhancer or activator of gene expression. An example of an enhancer capable of increasing gene expression is the AraC protein or the cAMP responsive protein (CRP). Preferably, the biomolecule is a

15 nucleic acid binding biomolecule. More preferably, the biomolecule is a nucleic acid binding (poly)peptide selected from the group consisting of regulator of transcription, regulator of translation, recombinase, (poly)peptide involved in RNA transport or a ribozyme capable of regulating transcription or translation. Even more preferred are (poly)peptides comprising the (poly)peptide sequence of tetracycline repressor; Lac

20 repressor; Xylose repressor; AraC protein; TetR-based T-Rex system (Yao et al., 1998, distributed by Invitrogen); erythromycin-specific repressor MphR(A) (Weber 2002); Pip (pristinamycin interacting protein, Fussenegger et al., 2000); ScbR (quorum sensing regulatory protein from *Streptomyces coelicolor*, Weber et al., 2003); TraR (quorum sensing regulatory protein of *Agrobacterium tumefaciens*);

25 fused to the eukaryotic activation domain p65 of NF κ B (Neddermann et al., 2003); chimeric proteins consisting of the: (i) Gal4 DNA-binding domain and either a full-length PhyA protein (PhyA-GBD) or the N-terminal photosensory domain of PhyB [PhyB(NT)-GBD] of *Arabidopsis thaliana* (Shimizu-Sato et al., 2002); (ii) steroid hormone regulated systems like the GeneSwitch regulatory system from Valentis

30 (www.geneswitch.com), sold by Invitrogen (catalog number K1060-02) and consisting of (1) a Gal4 DNA-binding domain fused to a human progesterone receptor ligand binding domain and an NF κ B-derived p65 eukaryotic transcription activation domain and (2) the inducer mifepristone; (iii) dimerizer systems from ARIAD consisting of (1) a ZFHD1 DNA-binding domain fused to FKBP 12, (2) a

35 modified form of FRAP, termed FRB, fused to the NF κ B-derived p65 activation domain and (3) rapamycin, AP22565 or AP12967 as heterodimer-forming agents, as they bind to both (1) and (2); (iv) Ecdysone-Inducible Expression System (with

5 the Inducing Agents Ponasterone A and Muristerone A) from Invitrogen (catalog
numbers K1001-01 K1003-01, K1004-01) containing (1) a modified form of the
Drosophila ecdysone receptor (VgEcR) fused to a VP16 activation domain, (2) the
mammalian homologue RXR of *ultraspiracle*, the natural binding partner of the
ecdysone receptor in *Drosophila* and (3) the inducers ponasterone A and
10 muristerone A.

The term "fragment" relates to the functional domains of a protein. These may be,
for example, a nucleic acid binding domain, a multimerization domain or an effector
domain. The person skilled in the art knows that such domains may be linked by
appropriate linkers. Although not every combination of domains may result in a
15 functional chimeric protein, the skilled person can easily determine whether a
specific chimeric protein has biological activity. In many cases no precise domain
boundaries may exist so that the functional domain may have additional N- and C-
terminal amino acid residues.

Nucleic acid binding (poly)peptides are known to have at least two states: in their
20 "off-state", nucleic acid binding (poly)peptides have a low affinity to their binding
sequence on the nucleic acid molecule, whereas in their "on-state", nucleic acid
binding (poly)peptides have a high affinity for their binding sequence. Accordingly,
when the biomolecule is a nucleic acid binding (poly)peptide, the term "modulating
the activity of a biomolecule" refers to the regulation of the nucleic acid binding
25 activity of the biomolecule. "Modulating the activity" can in some cases include, in
addition to "on-states" and "off-states", a number of intermediate states which
becomes particularly evident when the biomolecule is an allosterically regulated
protein, which are also comprised by the term "biomolecule" (see for example
Genes, Lewin, J. Wiley & Sons, 1983, 1st edition, page 222, 1st col., last paragraph
30 to 2nd col. last paragraph). The term "monitoring" means observe the activity or
expression level of a protein. "Monitoring" comprises direct or indirect measures.
Preferred are indirect measures which rely on an effect of the biomolecule on, for
example, transcription or translation of RNA. Particularly preferred are indirect
measures based on the expression of a reporter protein. In these cases, monitoring
35 means measuring the amount or activity of the reporter protein.

5 The term "host cell" means eukaryotic or prokaryotic cell. The cell can be the cell of
a uni-cellular or multi-cellular organism, which may be pathogenic or non-
pathogenic. Preferably, the host cell is selected from mammalian, insect, nematode,
plant, yeast, protist cell, gram-positive or gram-negative bacteria, archaebacteria or
protozoa. Preferably the cell is a cell expressing an allosterically regulated
10 biomolecule. More preferably, the cell is a cell expressing a biomolecule which is a
nucleic acid binding (poly)peptide. Said biomolecule can be expressed transiently
or, more preferable, stably.

The term "transforming", is used herein synonymous with transfection and means
introducing a nucleic acid molecule into a cell. Cells can be transformed by various
15 methods known in the art, including methods such as electroporation, lipofection,
viral infection, phage infection, microinjection. The term "introducing" refers to
method of transfecting or transforming a host cell with a nucleic acid molecule
encoding, for example, a (poly)peptide comprising an interaction partner of the
biomolecule. Introduction of the construct into the host cell can be effected by
20 calcium phosphate transfection, DEAE-dextran mediated transfection, cationic
lipid-mediated transfection, electroporation, transduction, infection or by other
methods. Such methods are described in many standard laboratory manuals, such
as Davis et al., Basic Methods In Molecular Biology (1986) or Sambrook et al.,
"Molecular Cloning, A Laboratory Manual"; ISBN: 0879695765, CSH Press, Cold
25 Spring Harbor, 2001.

The term "nucleic acid molecule" comprises DNA and RNA which may be single
stranded or double stranded, circular or linear. "Nucleic acid molecules encoding an
interaction partner" relates to expression constructs capable of mediating protein
expression. Said nucleic acid molecule introduced into the host cell comprises an
30 open reading frame encoding an interaction partner of said biomolecule in
expressible form. "Expressible form" implies that the nucleic acid molecule may
contain regulatory sequences that allow expression of the encoded (poly)peptide
upon introduction of the nucleic acid molecule into the host cell.

The nucleic acid molecule introduced into the host cell may be part of an expression
35 vector. Such vectors are usually specific for prokaryotic or eukaryotic expression.

- 5 However, the nucleic acid molecule may also be part of a vector functional in prokaryotes and eukaryotes.

A typical prokaryotic expression vector usually contains a promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription of the transcript. Transcription of
10 DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eukaryotic promoters differ from those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a prokaryotic system, and, further prokaryotic
15 promoters are not recognized and do not function in eukaryotic cells. Similarly, translation of mRNA in prokaryotes depends upon the presence of the proper prokaryotic signals which differ from those of eukaryotes. Promoters vary in their "strength" (i.e. their ability to promote transcription). In some cases it may be desirable to use strong promoters in order to obtain a high level of transcription and,
20 hence, expression of a protein. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when using *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *recA* promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lacUV5*,
25 *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene. Specific initiation signals are also required for efficient gene transcription and translation in
30 prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector or nucleic acid molecule encoding a (poly)peptide, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For
35 instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. The SD

5 sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, *Methods in Enzymology*, 68:473 (1979), which is hereby incorporated by reference. Thus, an SD-ATG combination that can be
10 utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the N gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used. Suitable expression vectors for
15 use in practicing the present invention include, for example, vectors such as pET expression vectors (Novagen), pQE expression vectors (Qiagen), pASK expression vectors (IBA), pBAD expression vectors (Invitrogen), pWH1950 (Ettner et al., 1996), pWH1520 (MoBiTec), pWH353 with a Xyl/Tet promoter (Geissendörfer and Hillen, 1990), pLZ vectors with SPAC, Xyl and Xyl/Tet promoter systems (Zhang et al.,
20 2000) or pNZ8008 with a *nisA* promoter (Eichenbaum et al., 1998).

A typical eukaryotic expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and
25 intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin, EF-1 α and ubiquitin C promoters). Suitable
30 expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Host cells that could be used include human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster
35 ovary (CHO) cells. Alternatively, the encoded (poly)peptides can be expressed in stable cell lines that contain a nucleic acid molecule of interest integrated into a

5 chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells. The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of
 10 interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., *Biochem J.* 227:277-279 (1991); Bebbington et al., *Bio/Technology* 10:169-175 (1992)). Using these markers, the cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and
 15 NSO cells are often used for the production of proteins. The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., *Molecular and Cellular Biology*, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites *Bam*HI, *Xba*I and *Asp*718, facilitate the
 20 cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene. As indicated above, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, Herpes simplex virus thymidine kinase, G418 or puromycin or zeocin resistance for eukaryotic cell culture and
 25 tetracycline, hygromycin, apramycin, zeocin, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Corynebacterium glutamicum* or *Bacillus subtilis*; fungal
 30 cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, 293 and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

The term "interaction partner of said biomolecule" refers to a string of amino acid
 35 residues with affinity to a (poly)peptide or aptamer, allosteric ribozyme, riboswitch or ribonucleoprotein. Said string of amino acid residues may be a peptide or a

5 polypeptide. Preferably, the string of amino acids with affinity to the biomolecule is part of a fusion protein. The interaction partner may be present at the carboxy terminal end of the fusion protein, it can, however, also be located at the amino terminal end or at an internal position of the amino acid sequence of the protein, provided that this is not associated with a negative property such as e.g. impeding
10 or destroying the biological activity etc. The larger subunit of the fusion protein can be a complete protein or a mutant of a protein such as e.g. a deletion mutant or substitution mutant. If the biomolecule is the Tet repressor or comprises (poly)peptide sequences of the Tet repressor, the interaction partner is preferably the polypeptide of the present invention or the polypeptide expressed by the nucleic acid molecule of the present invention or a fusion protein comprising said
15 (poly)peptide.

This is experimentally feasible, as different sets of more than 6000 yeast strains, each expressing a distinct GST-ORF, GFP-ORF or TAP-ORF fusion, have already been generated (Ghaemmaghami et al., 2003; Huh et al., 2003; Martzen et al.,
20 1999).

Components of tetracycline (tc)-dependent gene regulation systems are commonly used and have been introduced into almost every eukaryotic and prokaryotic model organism to conditionally control the expression of individual target genes. Thus, transgenic organisms and cell lines containing tc-dependent regulatory systems are
25 widely distributed among academic and industrial research groups. Typically, the expression of a gene is controlled by externally applying tc or one of its more active derivatives, doxycycline or anhydrotetracycline. Using phage display, as disclosed in the present invention, we have now identified an oligopeptide that, when expressed as a fusion with another protein like, for example, thioredoxin (Trx), can induce
30 expression of a gene under control of TetR *in vivo*. Such a peptide has not been described for TetR or any other allosteric regulatory protein. If the reporter gene under Tet control is sensitive and can be quantified over a wide expression range, like firefly luciferase, it is possible to quantify the expression of an endogenous protein that has been tagged with the peptide. If the reporter gene expressed is
35 green fluorescent protein, fluorescence activated cell sorting techniques can be used to separate cells that express the tagged protein from those that do not. In

5 bacteria, for example, this system can be used as a genetic reporter system to
address questions or problems in proteomics, once a collection of mutant strains
has been constructed that each contain a differently tagged protein, thus,
representing the entire proteome of the respective bacterium. The "ensemble" of
these strains can then be subjected to different growth conditions, like heat shock, N
10 source limitation, sporulation or the onset of stationary phase. The expression of the
reporter gene in an individual bacterium will reflect the expression pattern of the
respective "tagged" protein and the collection of tagged strains, thus, the "proteome"
of the respective condition. The intensity of the reporter gene signal correlates to the
expression level of the expressed and tagged protein. This allows the introduction of
15 genetic methods to the field of proteomics. According to the present state of the art,
this still requires protein-biochemical methods which are much more laborious and
time-consuming. Tagging approaches to detect protein expression visually by fusing
GFP to the C-terminus of an open reading frame (Huh et al., 2003) or to quantify
protein expression by fusing a "TAP" tag to the C-terminus of an open reading frame
20 (Ghaemmaghami et al., 2003) allow either separation of cells expressing a tagged
protein by FACS and quantification in the case of highly expressed proteins (GFP-
tag) or a precise quantification of a tagged proteins' expression level (TAP-tag).
Thus, quantification and visual detection requires the introduction of two
independent tags per protein.

25 The invention can be used to facilitate proteome analysis. First, a bacterial
population has to be generated that contains a set of individual bacteria that,
together, constitute the proteome of the organism. To achieve this, every single
bacterium in this population carries a single encoded protein that has been tagged
with the interacting peptide. In *Bacillus subtilis* or *Escherichia coli*, this would
30 correspond to about 4000 different strains. In other organisms, like *Streptomyces*
coelicolor, the number of strains needed would be about 7500, in *Mycoplasma*
pneumoniae only about 650. This is experimentally feasible, as a set of 6144 yeast
strains, each expressing a distinct GST-ORF fusion, has already been generated
(Martzen et al., 1999). Automated parallel approaches have allowed generating up
35 to 400 different expression vectors over a three day period. For an organism with a

5 genome about the size of yeast, this time-frame would extrapolate to a few months of work (Phizicky et al., 2003). (see figure 1)

Once the set of strains has been generated, the bacterial population carrying the tagged proteome can be used to explore the bacterial proteome. The bacterial population would be grown under different conditions, e. g. rich medium, minimal
10 medium, different growth temperatures, alternative carbon or nitrogen sources, logarithmic phase, stationary phase, exposure to different stresses like changes in the osmolarity, oxygen availability, different antibiotics or bacterial toxins, to name a few, but not necessarily all possible altered growth conditions. This could be done in an array-like setup in microtiter plates to facilitate identification of expressed
15 proteins or, more classically, on plates. Strains that express a tagged protein under the respective environmental conditions are identified by GFP fluorescence, either visually or under a fluorescence-microplate reader. The corresponding proteins are identified either by reading-out the position in the strain array or by direct sequencing of genomic DNA (ABI Prism 310 Genetic Analyzer User's Manual) using
20 the known DNA sequence of the tag. (see figures 2 and 20)

The teaching of the present application is particularly useful in disease proteomics. As of yet, proteome analysis of pathogenic microorganisms tries to mimic *ex vivo* the environmental conditions supposed to be encountered by the microorganism in the host [(*Streptococcus mutans* - (Len et al., 2003); *Salmonella typhimurium* -
25 (Deiwick et al., 2002); *Pseudomonas aeruginosa* - (Guina et al., 2003); *Leishmania infantum* - (El Fakhry et al., 2002); *Mycobacterium tuberculosis* - (Rosenkrands et al., 2002)]. *In vivo* analysis of pathogenicity-specific genes is mainly conducted by microarray analysis of mRNA expression (Chan et al., 2002; Eriksson et al., 2003; Okinaka et al., 2002; Staudinger et al., 2002) or analysis of bacterial strains
30 expressing different antisense constructs (Ji et al., 2001). The invention described here can facilitate the analysis of protein expression in the pathogenic organism in an *in vivo* animal disease model. The animal model is infected using one of the methods of the present invention by a bacterial population containing several copies of the tagged proteome. The disease is allowed to develop. At an appropriate
35 timepoint, the animal is sacrificed, the respective organs and tissues containing the pathogenic microorganism are isolated, bacterial translation is arrested by addition

5 of a bacteriostatic translation inhibitor like chloramphenicol and the tissue (if
necessary) is homogenized. Bacteria are isolated by filtration and sorted by FACS
(fluorescence activated cell sorting) into GFP-expressing and GFP-non-expressing
populations. These are sorted into single wells and streaked to clonal populations.
Due to the defined tag sequence, the individual protein that was expressed in the
10 host organism can then be identified by direct genomic sequencing.

The teaching of the present application is also particularly useful to facilitate
analysis of expression of proteins that are difficult to detect by conventional
proteomics. Low-abundance proteins like transcriptional regulators or signal
transducing proteins like kinases are often not detectable by standard proteome
15 methods, even though many of these proteins are important for pathogenesis or
development (Deiwick et al., 2002; Gygi et al., 2000). Induction of reporter gene
expression by binding of a tagged protein to TetR leads to amplification of the initial
signal by allowing multiple transcription events and subsequent multiple rounds of
translation per mRNA molecule.

20 Moreover, the teaching of the present application is also particularly useful to allow
exogenous control of endogenous riboswitch-controlled translational regulation
systems (Stormo, 2003). Riboswitches have been identified in all three kingdoms
(Sudarsan et al., 2003). Examples for molecules that bind to riboswitches are
thiamine (Winkler et al., 2002a), S-adenosylmethionine (Epshtein et al., 2003;
25 Winkler et al., 2003), FMN (Winkler et al., 2002b) and guanine (Mandal et al., 2003).
These are all common metabolites and their intracellular concentration cannot be
easily manipulated. Inducible expression of peptides that recognize these RNA
elements and can flip the switch would allow external control of central metabolic
pathways without having to interfere with the natural genetic situation through the
30 introduction of foreign control elements (repressors, activators) in classical
transcription control.

In a preferred embodiment of the present invention's method, the activity or degree
of modulation of the activity of the biomolecule is measured via a readout system.
The readout system can be based on the detection of a transcription product, a
35 translation product or on the activity of translated (poly)peptide. Readout system can

5 be any detectable product, the expression level of which is modulated by the action of the biomolecule. Readout may be a signal of a specific wavelength such as those generated by GFP or luciferase or the like. Also comprised by the present invention are enzyme based readout systems such as the CAT system. The readout system is controlled by a binding site for the regulatory biomolecule. For example, in the
10 case of a biomolecule with repressing activity, the binding site controlling the readout system is bound by said "biomolecule with repressing activity" and thereby expression from said readout system is blocked. Upon binding by the interaction partner disclosed in the present invention, the biomolecule is released and expression from the readout system is achieved.

15 In a more preferred embodiment of the present invention's method, (a) the readout system is provided by a nucleic acid molecule encoding a reporter protein; (b) the biomolecule is (i) a nucleic acid binding (poly)peptide selected from the group consisting of regulator of transcription, regulator of translation, recombinase, (poly)peptide involved RNA transport or (ii) an aptamer, allosteric ribozyme or
20 riboswitch and (c) the nucleic acid binding biomolecule is allosterically regulated. Aptamer, allosteric ribozyme or riboswitch are RNA molecules capable of performing a conformational switch after ligand binding. Peptide binding to RNA has been demonstrated by Rev-RRE (Gosser et al., 2001; Harada et al., 1996; Harada et al., 1999; Zhang et al., 2001) or Tar-TAT (Calnan et al., 1991; Weeks et al.,
25 1990), indicating that selection of peptides binding to a ligand binding site is a feasible approach. The principle of allosteric ribozymes is outlined in (Soukup and Breaker, 1999) and well known examples are hammerhead ribozymes dependent on the presence of ATP (Tang and Breaker, 1997), theophylline (Soukup et al., 2000) or cGMP/cAMP (Koizumi et al., 1999) for activity. Several examples of
30 riboswitches have been described in the literature. The best described is the vitamin B1-dependent *thiM* switch from *Escherichia coli* (Winkler et al., 2002a). Two examples of artificially generated ligand-controlled translational switches are described in (Suess et al., 2003) and (Werstuck and Green, 1998). The present invention particularly refers to the following RNA-binding (poly)peptides and their
35 recognition sequences which may be used in accordance with the teaching of the present invention and in any of the methods disclosed in the present invention: REV

- 5 binding peptide: clone 3-AAAAGRRARRRRRRRRRQSCRRKMTRD (Tan and Frankel, 1998); RRE site: 5'-UGGGCGCAGCGUCAUGACGCUGACGGUACA-3' (Peterson and Feigon, 1996); TAT peptide fragment: Tfr38-RKKRRQRRRPPQGSQTHQVSLSKQPTSQPRGDPTGPKE (Weeks et al., 1990); TAR site: 5'-
- 10 GGUCUCUCUGGUUAGACCAGAUCUGAGCCUGGGAGCUCUCUGGCUAACUAG AGAACCC-3' (Weeks et al., 1990); ATP-sensitive allosteric ribozyme: (i) ribozyme strand: 5'-
- 15 GGGCGACCCUGAUGAGUUGGGAAGAAACUGUGGCACUUCGGUGCCAGCAAC GAAACGGU-3'; (ii) substrate strand: 5'-GCCGUAGGUUGCCC-3' (Tang and Breaker, 1998); theophylline-sensitive allosteric ribozyme: cm⁺theo5: 5'-
- 20 GGGCGACCCUGAUGAGCCUGGAUACCAGCCGAAAGGCCCUUGGCAGUUAGA CGAAACGGUGAAAGCCGUAGGUUGCCC-3' (Soukup et al., 2000); cGMP-sensitive allosteric ribozyme: cGMP1: 5'-
- 25 GGGCGACCCUGAUGAGCCUUGCGAUGCAAAAAGGUGCUGACGACACAUCGA AACGGUGAAAGCCGUAGGUUGCCC-3' (Koizumi et al., 1999); Escherichia coli thiM riboswitch: 5'-
- 30 GGAACCAAACGACUCGGGGUGCCCUUCUGCGUGAAGGCUGAGAAAUACCCG UAUCACCUGAUCUGGAUAAUGCCAGCGUAGGGAAGUCACGGACCACCAGGU CAUUGCUUCUUCACGUUAUGGCAGGAGCAAACUAUGCAAGUCGACCUGCUG GAUCCAGCGCAA-3' (Winkler et al., 2002a); tetracycline-dependent translational switch: cb32 5'-
- CUUAAGGCCUGUACUGCUGCUUAAGGCCUAAAACAUACCAGAUCCGCCACCC GCGCUUUAUCUGGAGAGGUGAAGAAUACGACCACCUAGGCCAAAUGGCU AGC-3' (Suess et al., 2003); Hoechst33258-specific translational switch: H19 5'-
- 30 GGUGAUCAGAUUCUGAUCCAACAGGUUAUGUAGUCUCCUACCUCUGCGCCU GAAGCUUGGAUCCGUCGC-3' (Werstuck and Green, 1998);

The methods disclosed in the present invention allow to develop peptides or polypeptides capable of interacting with the above-mentioned biomolecules. In accordance with the present invention, it is required that the interacting

35 (poly)peptides not only bind to the biomolecules but also modulate their activity, preferably their nucleic acid binding affinity. For example by using phage display or

- 5 antibody libraries, new interaction partners of the Tet repressor might be identified which are capable of reducing the affinity of the Tet repressor for its recognition sequence. These newly identified sequences can either be expressed on their own or as part of a fusion protein and, thus, regulate the expression of the reporter protein located downstream of the recognition sequence of the Tet repressor.
- 10 Although only specific examples for interaction partners of Tet repressor are disclosed in the present invention, the skilled person can, based on the teaching of the present invention, identify modulators of any other nucleic acid binding protein. Generally, the interaction partner is a biological macromolecule. Preferably, new interaction partners are identified by using phage display or by screening antibody
- 15 libraries. Alternatively, interaction partners may be identified by ribozyme display, mRNA display, cell-surface display and/or lipocalin-based libraries. Peptide libraries based on these scaffolds (lipocalins, cell surface display, mRNA display) have been constructed, published and successfully used to select target-specific peptides. Phage display and combinatorial methods for generating interaction partners are
- 20 known in the art (as described in, e.g., Ladner et al. U.S. Pat. No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication
- 25 No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum Antibod Hybridomas 3:81-85; Huse et al. (1989) Science 246:1275-1281; Griffiths et al. (1993) EMBO J 12:725-734; Hawkins et al. (1992) J Mol Biol 226:889-896; Clackson et al. (1991) Nature
- 30 352:624-628; Gram et al. (1992) PNAS 89:3576-3580; Garrad et al. (1991) Bio/Technology 9:1373-1377; Hoogenboom et al. (1991) Nuc Acid Res 19:4133-4137; and Barbas et al. (1991) PNAS 88:7978-7982, the contents of all of which are incorporated by reference herein).

The basis for most phage display applications is the filamentous phage M13 from

35 *Escherichia coli*. A surface protein, gpIII which is present in five copies per phage and important for infection of the bacteria can tolerate N-terminal insertions to a

5 certain degree. Since the N-terminus of this protein is solvent-exposed, the
insertions are presented on the surface of the phage (Kay et al., 2001). The
commercially available phage bank Ph.D.-12TM from New England Biolabs is
preferably used to select for interaction partners. Alternatively, Ph.D.-7TM, Ph.D.-
C7CTM (New England Biolabs), FliTrx Random Peptide Display Library (Invitrogen),
10 Ready-To-Use Phage Display cDNA Libraries (Spring Bioscience) or pSKAN
Phagemid Display System (MoBiTec) may be used. Phage bank Ph.D.-12TM
contains $\sim 10^9$ different dodecapeptides fused via a flexible linker of four amino acids
to the N-terminus of the gpIII protein. The *in vitro* selection may be performed by
coating polystyrene tubes (NUNC Maxisorb) with purified biomolecule. Generally,
15 the tubes are then incubated with the pool of M13 phages, washed several times
and phages are eluted either specifically by addition of biomolecule or unspecifically
by lowering the pH value. Individual M13 clones are usually picked and sequenced
after three selection rounds.

Binding of individual phage clones to the biomolecule may be determined by using
20 an ELISA (Enzyme Linked Immunosorbent Assay). The phages are amplified,
precipitated and resuspended in a small volume. Subsequently, 96-well microtiter-
plates may be coated with the biomolecule, then blocked with a blocking reagent
such as Bovine Serum Albumin, followed by incubation with increasing amounts of
M13 phages from different isolates, several washes with buffer and, finally,
25 incubated with a phage-specific monoclonal antibody covalently coupled to
horseradish peroxidase. Addition of ABTS (2', 2'-Azino-bis(ethylbenzthiazolin-6-
sulfonic acid) as substrate permits the spectrophotometric detection of phage-binding
to TetR. The degree of absorption thereby serves as a quantitative indicator of
phages bound to the target protein (Kay et al., 2001).

30 Repressor-inducing interaction partners may be isolated as discussed below. Since
small oligopeptides are rapidly degraded intracellularly by proteases, the peptide-
encoding sequences may be cloned as C-terminal fusions to the *Escherichia coli*
protein thioredoxin, an established carrier protein for peptides (Park and Raines,
2000). The thioredoxin fusion proteins can be expressed, for example, by using a
35 plasmid containing a *tac* promoter under control of Lac repressor (Ettner et al.,
1996). The biomolecule of interest may be expressed constitutively at a low level.

5 Preferably, in particular if the regulatory biomolecule is the Tet repressor, the indicator strain is *E. coli* DH5 α (λ *tet50*) containing the phage λ *tet50* (Smith and Bertrand, 1988) integrated in single copy into the *E. coli* genome. This phage contains a *tetA-lacZ* transcriptional fusion. Expression of β -galactosidase is, thus, regulated by TetR that binds to *tetO* sequences located within the promoter. The
10 pool of potential interaction partners is screened, for example, by plating transformed colonies on MacConkey agar containing IPTG. When using the above-indicated expression vector under suitable conditions, an inducing interaction partner of the biomolecule, in this case of the Tet repressor, will lead to the expression of β -galactosidase, resulting in an acidification of the medium
15 surrounding the colony which can be detected by its yellow color.

Preferably, individual candidates identified by sequencing are cloned as fusion proteins, transformed into suitable reporter strains. For example, if the repressor is the Tet repressor, the bacterial strain DH5 α (λ *tet50*) may be used and the respective β -galactosidase activity is subsequently determined. For example, if the repressor is
20 the Tet repressor, the following controls might also be included in the measurements: To define the regulatory window, both the repressed state (0% - TetR binds to *tetO*) and the fully induced state in the presence of tc (100% - TetR dissociates from *tetO*) may be determined. To exclude that the remainder of the fusion protein, i.e. the carrier protein, interacts unspecifically, i.e. by amino acid
25 residues of the carrier protein and not by amino acid residues of the interaction partner, a plasmid expressing the carrier protein without a peptide fusion may also be assayed in the presence and absence of IPTG. The β -galactosidase activities of the individual candidates cloned can also determined in the presence and absence of IPTG.

30 Interaction sites between the interaction partner and the biomolecule may be determined by epitope mapping. Particularly preferred is the isolation of the interaction site by *in vivo* epitope mapping, taking advantage of the observation that TetR(B/D) is not induced by the peptide. Chimeric repressor molecules may be constructed in which *tetR*(B) sequences are exchanged to different extents by the
35 corresponding sequences from *tetR*(D) (Schnappinger et al., 1998). *In vivo*

5 inducibility is determined by TrxA-pepBs1. In the case of Tet repressor, the results of inducibility profiles show that interactions between repressor and peptide are confined to the region from helix $\alpha 8$ to residue 182 in helix $\alpha 10$ (see table 1). The loop connecting the helices $\alpha 8$ and $\alpha 9$ also appears to be important, as a chimera containing *tetR*(D) sequences at residues 153–167 is not inducible by TrxA-pepBs1.
 10 The loop between helices $\alpha 9$ and $\alpha 10$ also seems to be important, as chimeras containing *tetR*(D) sequences between residues 179-184 and 180-184 are not inducible by TrxA-pepBs1.

In particular cases it may be desirable to increase or decrease the strength of the interaction between the biomolecule and the interacting peptide or polypeptide. In
 15 these cases, the interaction can be modified by

(i) altering the fusion point of the peptide with the target protein. For thioredoxin, an N-terminal fusion induces TetR more sensitively than a C-terminal one (Figure 17),

(ii) by altering the expression level of TetR. TetR is expressed constitutively to a higher level from a pWH1411-derivative than from a pWH510-derivative (Wissmann
 20 et al., 1991). Consequently, higher amounts of N-terminally peptide-tagged thioredoxin are needed for full induction of β -galactosidase activity (Figure 16) and/or by

(iii) selectively exchanging, adding or deleting amino acid residues within the interacting (poly)peptide or the biomolecule. Optimal interaction may be achieved by
 25 using a rational design or by randomizing particular subunits of the (poly)peptide or biomolecule. Rational design of interaction partners may first require to obtain structural information of the interacting (poly)peptide and the biomolecule or to use available structural information. For example, by using the three-dimensional information available for the Lac repressor in a method of computer modeling, new
 30 peptide sequences can be identified, capable of binding to and inducing a conformational change in the Lac repressor which will then modify the nucleic acid binding affinity of the Lac repressor. On the other hand, known modulators of the biomolecules might be used in any of the methods of the present invention. Examples of proteins as cofactor of a regulator include phosphorylated Hpr and
 35 CcpA from *Bacilli* (Aung-Hilbrich et al., 2002), TnrA and feedback-inhibited

5 glutamine synthetase from *Bacillus subtilis* (Wray et al., 2001), Mlc and dephosphorylated PtsG (Lee et al., 2000), or MalT and MalY (Schreiber et al., 2000) from *Escherichia coli*. Moreover, known interaction partners of regulatory biomolecules may be expressed, for example in a phage display library, wherein portions of their sequence are randomized. This will allow to isolate new interaction
10 partners with modified properties, either binding more tightly or less tightly to the biomolecule.

In a preferred embodiment of the present invention's method, the transformed nucleic acid molecule encoding the interaction partner of the regulatory biomolecule is a nucleic acid molecule comprising: (a) a nucleic acid sequence encoding a
15 peptide tag or a tagged (poly)peptide, operatively linked to expression control sequences; (b) a nucleic acid sequence encoding a tagged (poly)peptide or a peptide tag, a selectable marker gene and additional nucleotide sequences for site specific, in-frame integration of said nucleic acid molecule into the coding sequence of at least one host (poly)peptide of interest, or (c) a nucleic acid sequence
20 encoding a peptide tag, a selectable marker gene and additional nucleotide sequences for transposase-mediated random integration of said nucleic acid molecule into the coding sequence of at least one host (poly)peptide of interest. Constructs containing a nucleic acid sequence encoding a tagged (poly)peptide or a peptide tag, a selectable marker gene and additional nucleotide sequences for site
25 specific, in-frame integration of said nucleic acid molecule into the coding sequence of at least one host (poly)peptide of interest are particularly useful for generating host cells expressing tagged host proteins. Such constructs allow in-frame integration of tag encoding sequences into chromosomal sequences encoding, for example, a host protein. A DNA module that begins with the interaction tag-
30 encoding sequence and includes a both excisable and selectable marker (present on plasmids like pKD3, pKD4, pKD13 (Datsenko and Wanner, 2000)) may be amplified by a standard polymerase chain reaction with primers that carry extensions homologous to the C-terminal end of the targeted gene and to a region downstream of it in the host genome. Transformation of a strain expressing
35 bacteriophage λ red functions [encoded, for example, on a plasmid like pKD20 (Datsenko and Wanner, 2000)] yields recombinants carrying the targeted gene fused to the interaction tag-encoding sequence [(Uzzau et al., 2001); a modification of a method originally described by Datsenko and Wanner (2000)]. The selectable

5 marker may be, but is not limited to, an expression cassette conferring resistance against an antibiotic like ampicillin, apramycin, chloramphenicol, erythromycin, hygromycin, kanamycin, tetracycline, or zeozin. It is flanked by recognition sites for site-specific recombinases, like FRT/Flp [expressed from a plasmid like pCP20 (Cherepanov and Wackernagel, 1995)] or loxP/Cre. The PCR primers are between
 10 56 to 60 nucleotides long, (i) anneal to constant regions of 20 to 21 nucleotides of the amplification template and (ii) carry extensions of 36 to 40 nucleotides that are identical in sequence to the last portion of the gene to be targeted (downstream primer) and to a region downstream of the gene (upstream primer). The PCR fragments are purified and introduced into the strain of interest and selected for
 15 recombinants carrying the amplified sequence in the genome. As a result of this targeted insertion, the host protein contains an N-terminal, C-terminal or internal amino acid sequence capable of interacting with the regulatory biomolecule.

Another method to introduce the amino acid sequence capable of interacting with the regulatory biomolecule is to integrate it into a transposable genetic element.
 20 Transposable genetic elements are remarkably diverse molecular tools for both whole-genome and single-gene studies in bacteria, yeast, and other organisms. Efficient, but simple transposon-based signature-tagged mutagenesis and genetic footprinting strategies have pinpointed essential genes and genes that are crucial for the infectivity of a variety of human and other pathogens. Individual proteins and
 25 protein complexes can be dissected by transposon-mediated scanning linker mutagenesis (Hamer et al., 2001; Hayes, 2003; Judson and Mekalanos, 2000). Such a DNA module would include two inverted repeats at the ends of the construct which serve as recognition sites for the respective transposase, the interaction-tag sequence separated by a linker sequence from one of the inverted repeats and an
 30 excisable selectable marker. The DNA element may be generated from a plasmid template by amplification via a standard polymerase chain reaction or by cleavage with a restriction enzyme. It is then incubated in vitro with the respective transposase molecule and introduced by standard transformation techniques into the cell of interest, where it integrates randomly into the host genome (Goryshin et al., 2000). Alternatively, it can be introduced by in vivo methods reviewed in Berg,
 35 C.M. & Berg, D.E. (1996), pp. 2588–2612, in *Escherichia coli* and *Salmonella*: cellular and molecular biology (eds. Neidhardt, F.C. et al.) (ASM Press, Washington, DC). Recombinants are selected for by the marker, which may be, but is not limited to, an expression cassette conferring resistance against an antibiotic like ampicillin,

5 apramycin, chloramphenicol, erythromycin, hygromycin, kanamycin, streptomycin, tetracycline, or zeocin. The marker may also be an expression cassette conferring dependence on the antibiotic streptomycin for growth (Gregory et al., 2001). The marker is flanked by recognition sites for site-specific recombinases, like FRT/Flp or loxP/Cre. Roughly, one sixth of the recombinants obtained will contain an insertion
 10 of the interaction-tag encoding sequence leading to an in-frame protein fusion with an endogenous ORF. Upon expression of the host protein, the biomolecule will interact with the tagged host protein. In cases when the biomolecule is a nucleic acid binding (poly)peptide, this interaction results in a modulation of the biomolecules' affinity to its recognition sequence. For example a repressor might be
 15 switched into its inactive state and thereby allow transcription of the gene controlled by the repressor.

As outlined above, the interaction partner of the biomolecule may be a peptide tag or a tagged (poly)peptide. A tagged (poly)peptide is a fusion protein containing amino acid residues capable of interacting with the biomolecule. Preferred fusion
 20 peptides are those encoded by the nucleic acid molecules of the present invention, in particular when the biomolecule is the Tet repressor or comprises a fragment of the Tet repressor. The interaction partner may be located at N- or C-terminal position of the fusion peptide or may be located at an internal position, provided that the activity of the biomolecule is not affected by the presence of the interaction
 25 partner. In some cases, it may be desirable to add the interaction partner externally, i.e. by adding, for example, the peptide or polypeptide to the culture medium of the cell. In these cases the interaction partner also may be a peptide attached to a carrier polypeptide. Preferably, said attachment is a covalent attachment, for example achieved by crosslinking the interacting peptide with the carrier polypeptide.
 30 The conditions and reagents required for crosslinking are known to the person skilled in the art. A comprehensive product guide and reagents for crosslinking are, for example, obtainable from Pierce Biotechnology, Inc., P.O. Box 117, Rockford, IL, 61105, USA.

In another preferred embodiment of the present invention, the nucleic acid binding
 35 (poly)peptide comprises the (poly)peptide sequence of (a) Tet repressor; (b) Lac repressor; (c) Xylose repressor; (d) AraC protein; (e) TetR-based T-Rex system; (f) erythromycin-specific repressor MphR(A); (g) Pip (pristinamycin interacting protein);

- 5 (h) ScbR from *Streptomyces coelicolor*, (i) TraR of *Agrobacterium tumefaciens*, fused to the eukaryotic activation domain p65 of NFκB; (j) chimeric proteins consisting of the: (i) Gal4 DNA-binding domain and either a full-length PhyA protein (PhyA-GBD) or the N-terminal photosensory domain of PhyB [PhyB(NT)-GBD] of *Arabidopsis thaliana*; (ii) steroid hormone regulated systems like the GeneSwitch
- 10 regulatory system from Valentis (www.geneswitch.com), sold by Invitrogen (catalog number K1060-02) and consisting of (1) a Gal4 DNA-binding domain fused to a human progesterone receptor ligand binding domain and an NFκB-derived p65 eukaryotic transcription activation domain and (2) the inducer mifepristone; (iii) dimerizer systems from ARIAD consisting of (1) a ZFHD1 DNA-binding domain
- 15 fused to FKBP 12, (2) a modified form of FRAP, termed FRB, fused to the NFκB-derived p65 activation domain and (3) rapamycin, AP22565 or AP12967 as heterodimer-forming agents, as they bind to both (1) and (2); (iv) Ecdysone-Inducible Expression System (with the Inducing Agents Ponasterone A and Muristerone A) from Invitrogen (catalog numbers K1001-01 K1003-01, K1004-01)
- 20 containing (1) a modified form of the *Drosophila* ecdysone receptor (VgEcR) fused to a VP16 activation domain, (2) the mammalian homologue RXR of *ultraspiracle*, the natural binding partner of the ecdysone receptor in *Drosophila* and (3) the inducers ponasterone A and muristerone A. Particular examples of (poly)peptides which may be used in accordance with the teaching of the present invention are the
- 25 following (poly)peptides: (a) **Tet repressor**: GenBank accession number: X00694 MSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAI EMLDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYE TLENQLAFLCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSM PPLLRQAIELFDHQGAEP AFLFGLELIICGLEKQLKCESGS; (a-1) **tTA** (TetR-VP16)
- 30 (Gossen and Bujard, 1992): MSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAI EMLDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYE TLENQLAFLCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSM PPLLRQAIELFDHQGAEP AFLFGLELIICGLEKQLKCESGSAYSRRARTKNNYGSTIE
- 35 GLLDLPDDDAPEEAGLAAPRLSFLPAGHTRRLSTAPPTDVSLGDELHLDGEDVAM AHADALDDFDLDMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDE YGG; (a-2) **tTA2** (TetR-FFF) (Baron et al., 1997):

5 MSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAI
 EMLDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYE
 TLENQLAFLCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSM
 PLLRQAIELFDHQGAEP AFLFGLELIICGLEKQLKCESGGPADALDDFDLDMLPAD
 ALDDFDLDMLPADALDDFDLDMLPG; (a-3) **tTA-p65** (TetR-p65) (Urlinger et al.,
 10 2000):
 MSRLDKSKVINSALELLNEVGIEGLTTRKLAQKLGVEQPTLYWHVKNKRALLDALAI
 EMLDRHHTHFCPLEGESWQDFLRNNAKSFRCALLSHRDGAKVHLGTRPTEKQYE
 TLENQLAFLCQQGFSLENALYALSAVGHFTLGCVLEDQEHQVAKEERETPTTDSM
 PLLRQAIELFDHQGAEP AFLFGLELIICGLEKQLKCESGSSEFQYLPDTDDRHRIE
 15 EKRKRTYETFKSIMKKSPFSGPTDPRPPRRRIAVPSRSSASVPKPAPQPYPFTSSL
 STINYDEFPTMVFP SGQISQASALAPAPPQVLPQAPAPAPAPAMVSALAQAPAPVP
 VLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDDEDLGALLGNSTDPAVFTDL
 ASVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAPAPLGAP
 GLPNGLLSGDEDFSSIADMDFSALLSQISS; (b) **Lac repressor**: GenBank
 20 accession number: J01636:
 MKPVTLYDVAEYAGVSYQTVSRVVNQASHVSAKTREKVEAAMAELNYIPNRVAQQ
 LAGKQSLLIGVATSSLALHAPSQIVAAIKSRADQLGASVVSMVERSGVEACKAAV
 HNLLAQRVSGLIINYPLDDQDAIAVEAACTNVPALFLDVSDQTPINSIIFSHEDGTRL
 GVEHLVALGHQQIALLAGPLSSVSARLRLAGWHKYLTRNQQIPIAEREGDWSAMS
 25 GFQQTMMQMLNEGIVPTAMLVANDQMALGAMRAITESGLRVGADISVVGYYDDTEDS
 SCYIPPSTTIKQDFRLLGQTSVDRLLQLSQGQAVKGNQLLPVSLVKRKTTLAPNTQ
 TASPRLADSLMQLARQVSRLESGQ; (c) **Xylose repressor**: GenBank accession
 number: NC000964:
 MTGLNKSTVSSQVNTLMKESMVFEIGQGQSSGGRRPVMLVFNKKAGYSVGIDVG
 30 VDYINGILTDLEGTIVLDQYRHLESNSPEITKDILIDMIHHFITQMPQSPYGFIGIGICV
 PGLIDKDQKIVFTPNSNWRDIDLKSSIQEKYNVSVFIENEANAGAYGEKLFGAAKNH
 DNIIYVSISTGIGIGVIINNHLYRGVSGFSGEMGHMTIDFNGPKCSCGNRGCWELYA
 SEKALLKSLQTKEKKLSYQDIINLAHLNDIGTLNALQNFGFYLGIGLTNINLNTFNPQAV
 ILRNSIIESHPMVLNSMRSEVSSRVYSQLGNSYELLPSSLGQNAPALGMSSIVIDHF
 35 LDMITM;

- 5 (d) **AraC protein:** GenBank accession number: J01641
 MAEAQNDP LLPGYSFNAHLVAGLTPIEANGYLDF FIDRPLGMKGYILNLTIRGQG WV
 KNQGREFVCRPGDILLFPPGEIHHYGRHPEAREWYHQWVYFRPRAYWHEWLNW
 PSIFANTGFFRPDEAHQPHFSDLFGQIINAGQGEGRYSELLAINLLEQLLLRRMEAI
 NESLHPPMDNRVREACQYISDHLADSNFDIASVAQHVCLSPSRLSHLFRQQLGISV
 10 LSWREDQRISQAKLLLSTTRMPIATVGRNVGFDDQLYFSRVFKKCTGASPSEFRA
 GCEEKVNDVAVKLS; (e) **TetR-based T-Rex system:** the TetR sequence is
 identical to that in (a);
- (f) **repressor protein MphR(A):** GenBank accession number: AB038042:
 MPRPKLKSDDEVLEAATVVLKRCGP IEF T LSGVAKEVGLSRAALIQRF TNRDTLLVR
 15 MMERGVEQVRHYLNAIPIGAGPQGLWEFLQVLVRSMNTRNDFSVNYLISWYELQV
 PELRTLAIQRNRAVVEGIRKRLPPGAPAAAELLHLSVIAGATMQWAVDPDGELADH
 VLAQIAAILCLMFPEHDDFQLLQAHA; (g) ***Streptomyces coelicolor***
transcriptional regulator Pip: GenBank accession number AF193856
 MMSRGEVRMAKAGREGPRDSVWLSGEGRRGRRGRQPSGLDRDRITGVTVRLL
 20 DTEGLTGFSMHRLAAELNVTAMSVYWYVDTKDQLLELALDAVFGELRHPDPDAGL
 DWREELRALAREN RALLVRHPWSSRLVGTYLNIGPHSLAFSRAVQNVVRRSGLPA
 HRLTGAISAVFQFVYGYGTIEGRFLARVADTGLSPEEYFQDSMTAVTEVPDTAGVI
 EDAQDIMAARGGDTVAEMLDRDFEFALDLLVAGIDAMVEQA; (h) ***Streptomyces***
***coelicolor* transcriptional regulator ScbR:** GenBank accession number
 25 AJ007731:
 MAKQDRAIRTRQTILDAAAQVFEKQGYQAATITEILKVAGVTKGALYFHFQSKEELA
 LGVFDAQEPPQAVPEQPLRLQELIDMGMLFCHRLRTNVVARAGVRLSMDQQAHG
 LDRRGPFRRWHETLLKLLNQAKENGELLPHVVT TDSADLYVGTFAGIQVVSQTVS
 DYQDLEHRYALLQKHILPAIAVPSVLAALDLSEERGARLAAELAPTGKD; (i) **TraR-**
 30 **p65 fusion (Neddermann et al., 2003):**
 MEFQYLPDTDDRHRIEEKRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSA
 SVPKPAPQPYPFTSSLSTINYDEFPTMVFP SGQISQASALAPAPPQVLPQAPAPAP
 APAMVSALAQAPAPVPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDD EDL
 GALLGNSTDP AVFTDLASVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTG
 35 AQRPPDPAPAPLGAPGLPNGLLSGDEDFSSIADMDFSALLSQISSGSARGVPPKKK
 RKGIGIQEGISAASRSMQHWLDKLTDLAAIEGDECILKTGLADIADHFGFTGYAYLHI

5 QHRHITAVTNYHRQWQSTYFDKKFEALDPVVKRARSRKHIFTWSGEHERPTLSKD
 ERAFYDHASDFGIRSGITIPKTANGFMSMFTMASDKPVIDLDREIDAVAAAATIGQI
 HARISFLRTTPTAEDAACVDPKEATYLRWIAVGKTMEEIADVEGVKYNSVRVKLRE
 RMKRFDVRSKAHLTALAIRRKLI; (j-i) The skilled person knows that almost any
 fusion of the Phy sequences to a Gal4-DBD (aa's 1-63, 1-95, 1-141) would be
 10 active. (j-ii) **Gal4-hpr-p65** from pSwitch (Invitrogen: Geneswitch_man.pdf):
 MDSQQPDLKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPL
 TRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLEFPGVDQKKFNKV
 RVVRALDAVALPQPVGVPNESQALSQRFTFSPGQDIQLIPPLINLLMSIEPDVIYAG
 HDNTKPDTSSSLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSL
 15 MVFGLGWRSYKHVSGQMPLYFAPDLILNEQRMKESSFYSLCLTMWQIPQEFVKLQV
 SQEEFLCMKVLLLLNTIPLEGLRSQTQFEEMRSSYIRELIKAIGLRQKGVVSSSQRF
 YQLTKLLDNLHDLVKQLHLYCLNTFIQSRALSVEFPMMSEVIAGSTPMEFQYLPDT
 DDRHRIEEKRKRTYETFKSIMKKSPFSGPTDPRPPPRRIAVPSRSSASVPKPAPQP
 YPFTSSLSTINYDEFPTMVFPSPGQISQASALAPAPPQVLPQAPAPAPAPAMVSALA
 20 QAPAPVPVLAPGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDDEDLGALLGNSTD
 PAVFTDLASVDNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAP
 APLGAPGLPNGLLSGDEDFSSIADMDFSALLSQISS; (j-iii-1) **ZHFD1-FKBP fusion**
 (www.ariad.com/regulationkits)
 MDYPAAKRVKLDSESRERPYACPVESCDRRFSRSEDELTRHIRIHTGQKPFQCRICMR
 25 NFSRSDHLTTHIRHTGGGRRRKKRTSIETNIRVALEKSFLENQKPTSEEITMIADQL
 NMEKEVIRVWFCNRRQKEKRINTRGVQVETISPGDGRTFPKRGQTCVVHYTGMLE
 DGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGAT
 GHPGIIPPHATLVFDVELLKLEVEGVQVETISPGDGRTFPKRGQTCVVHYTGMLE
 GKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATG
 30 HPGIIPPHATLVFDVELLKLETRGVQVETISPGDGRTFPKRGQTCVVHYTGMLE
 KKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGH
 PGIIPPHATLVFDVELLKLETSY; (j-iii-2) **FRB-p65 fusion**
 (www.ariad.com/regulationkits):
 MDYPAAKRVKLDSESRILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMER
 35 GPQTLKETSFNQAYGRDLMEAEWCRKYMKSGNVKDLLQAWDLYYHVFRISK
 RDEFPTMVFPSPGQISQASALAPAPPQVLPQAPAPAPAPAMVSALAQAPAPVPVLA
 PGPPQAVAPPAPKPTQAGEGTLSEALLQLQFDDDEDLGALLGNSTDPAVFTDLASV

5 DNSEFQQLLNQGIPVAPHTTEPMLMEYPEAITRLVTGAQRPPDPAPAPLGAPGLPN
 GLLSGDEDFSSIADMDFSALLSQISSTSY; (j-iv-1) **VgEcR** from pVgRXR
 (<http://www.invitrogen.com/content/sfs/vectors/pvgrxr.pdf>):

MAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPHDSAPY
 GALDMADFEFEQMFTDALGIDEYGGKLLGTSTRRISNSISSGRDDLSPSSSLNGYSA
 10 NESCDAKKSKKGPAPRVQEELCLVCGDRASGYHYNALTCGSCKVFFRRSVTKSA
 VYCKFGRACEMDMYMRRKCQECRLKKCLAVGMRPECVVPENQCAMKRREEKA
 QKEKDKMTTSPSSQHGGNGSLASGGGQDFVKKEILDMTCEPPQHATIPLLPDEIL
 AKCQARNIPSLTYNQLAVIYKLIWYQDGYEQPSEEDLRRIMSQPDENESQTDVSFR
 HITEITILTVQLIVEFAKGLPAFTKIPQEDQITLLKACSSEVMMLRMARRYDHSSDSIF
 15 FANNRSYTRDSYKMAGMADNIEDLLHFCRQMFMSMKVDNVEYALLTAIVIFSDRPGL
 EKAQLVEAIQSYIDTLRIYILNRHCGDSMSLVFYAKLLSILTELRTLGNQNAEMCFS
 LKLKNRKLPKFLEEIWDVHAIPPSVQSHLQITQEENERLERASVGGAITAGI
 DCDSASTSAAAAAAQHQPQPQPQPSSLTQND SQHQTQPQLQPQLPPQLQGQ
 LQPQLQPQLQTQLQPQIQPQPQLLPVSAPVPASVTAPGSLSAVSTSSEYMGGSA
 20 IGPITPATTSSITA AVTASSTTS AVPMGNGVGVGVGVGGNVSMYANAQTAMALMG
 VALHSHQEQLIGGVAVKSEHSTTA; (j-iv-2) **RXR** from pVgRXR
 (<http://www.invitrogen.com/content/sfs/vectors/pvgrxr.pdf>):

MDTKHFLPLDFSTQVNSSLTSPTGRGSMAAPSLHPSLGPGIGSPGQLHSPISTLSS
 PINGMGPPFSVISSPMGPHSMSVPTTPTLGFSTGSPQLSSPMNPVSSSEDIKPPLG
 25 LNGVLKVPAPSGNMA SFTKHICAICGDRSSGKH YGVYSCEGCKGFFKRTVRKDL
 TYTCRDNKDCLIDKRQRNRCQYCRYQMCLAMGMKREAVQEERQRGKDRNENEV
 ESTSSANEDVPVERILEAELAVEPKTETYVEANVGLNPSSPNDPVTNICQAADKQL
 FTLVEWAKRIPHFSSELPLDDQVILLRAGWNELLIASFSHRSIAVKD GILLATGLHVHR
 NSAHSAGVG AIFDRVLTELVSKMRDMQMDKTELGCLRAIVLFNPDSKGLSNPAEV
 30 EALREKVYASLEAYCKHKYPEQPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGDTPI
 DTFLMEMLEAPHQMT.

Particularly preferred are active fragments of said (poly)peptides.

In yet another preferred embodiment of the present invention, the reporter protein of
 the readout system is β -galactosidase, CAT, β -glucuronidase; β -xylosidase; Xyle
 35 (catechol dioxygenase); TreA (trehalase); GFP and variants CFP, YFP, EGFP,

- 5 GFP+ (Scholz et al., 2000) of GFP; bacterial luciferase (*luxAB*); *Photinus* luciferase; *Renilla* luciferase; coral-derived photoproteins including DsRed, HcRed, AmCyan, ZsGreen, ZsYellow, AsRed; alkaline phosphatase or secreted alkaline phosphatase.

In a preferred embodiment of the present invention's method, the reporter protein is a protein that confers resistance to an antibiotic. In this case, it is preferred that the
 10 cells be cultivated in the presence of an antibiotic so that only clones expressing the reporter protein are capable of propagating. Generally, the protein can mediate resistance to an antibiotic such as Ampicillin, Chloramphenicol, G418, Gentamycin, Hygromycin B, Kanamycin, Methotrexate, Neomycin, Streptomycin, Tetracycline, Tobramycin, or Vancomycin. Further examples of antibiotics are Penicillins:
 15 Ampicillin HCl, Ampicillin Na, Amoxycillin Na, Carbenicillin disodium, Cephalosporins, Cefotaxim Na, Cefalexin HCl, Cycloserine Penicillin G. Other examples include bacteriostatic inhibitors such as: Chloramphenicol, Erythromycin, Lincomycin, Tetracycline, Spectinomycin sulfate, Clindamycin HCl, Chlortetracycline HCl. Additional examples are proteins that allow selection with bactericidal inhibitors
 20 such as those affecting protein synthesis irreversibly causing cell death. Aminoglycosides can be inactivated by enzymes such as NPT II which phosphorylates 3'-OH present on kanamycin, thus inactivating this antibiotic. Some aminoglycoside modifying enzymes acetylate the compound and block their entry in to the cell. Proteins that allow selection with nucleic acid metabolism inhibitors like
 25 Rifampicin, Mitomycin C, Nalidixic acid, Doxorubicin HCl, 5-Fluorouracil, 6-Mercaptopurine, Antimetabolites, Miconazole, Trimethoprim, Methotrexate, Metronidazole, Sulfamethoxazole are also examples for reporter proteins.

In a preferred embodiment of the present invention the cell or host cell is selected from a mammalian, insect, nematode, plant, yeast, protist cell, non-pathogenic
 30 Gram-positive or Gram-negative bacteria, non-pathogenic archaeobacteria or non-pathogenic protozoa. In another preferred embodiment of the present invention the cell is a pathogenic organism selected from the group consisting of yeast, Gram-positive bacteria, Gram-negative bacteria, archaeobacteria or protozoa.

In yet another preferred embodiment of the present invention, all or a subset of the
 35 proteins encoded by the cell are tagged. Tagged proteins may be expressed as

5 stable construct, i.e. integrated into the genome of the cell or transiently from a vector. Preferably, the tagged protein is expressed from its natural position in the genome of the respective organism.

The present invention also relates to a method of producing and/or selecting a compound capable of modulating a nucleic acid binding protein comprising the
10 steps of: (a) conducting a selection of compounds with the nucleic acid binding target protein under conditions allowing an interaction of the compound and the nucleic acid binding protein; (b) removing unspecifically bound compounds; (c) detecting specific binding of compounds to the nucleic acid binding target protein; (d) expressing in a cell, the nucleic acid binding protein and providing *in trans* the
15 coding sequence of at least one indicator gene, wherein said coding sequence is under control of the target sequence of the nucleic acid binding protein; (e) adding a candidate compound to the cell of step (d); (f) determining the amount or activity of the indicator protein, wherein a reduced or increased amount of indicator protein is indicative of compounds capable of modulating the activity of the nucleic acid
20 binding protein; and (g) selecting compounds capable of modulating the activity of the nucleic acid binding protein. The compound identified by this method is an interaction partner of said biomolecule. Preferably, said compounds are (poly)peptides or derivatives thereof.

In a preferred embodiment of the present invention, particularly in cases when the
25 biomolecule is the Tet repressor, said compound is any of the (poly)peptides encoded by the nucleic acid molecules of the present invention or a derivative thereof. Derivatives may be (poly)peptides with substitutions, deletions or additions in order to improve the (poly)peptide's affinity and/or specificity to the biomolecule. In addition, said compounds may contain chemical modifications in order to improve
30 the solubility and/or cellular uptake of the compound. Such modifications include, but are not limited to (i) esterification of carboxyl groups, or (ii) esterification of hydroxyl groups with carbon acids, or (iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or (iv) formation of pharmaceutically acceptable salts, or (v) formation of pharmaceutically acceptable
35 complexes, or (vi) synthesis of pharmacologically active polymers, or (vii) introduction of hydrophilic moieties, or (viii) introduction/exchange of substituents on

- 5 aromates or side chains, change of substituent pattern, or (ix) modification by introduction of isosteric or bioisosteric moieties, or (x) synthesis of homologous compounds, or (xi) introduction of branched side chains, or (xii) conversion of alkyl substituents to cyclic analogues, or (xiii) derivatisation of hydroxyl groups to ketals, acetals, or (xiv) N-acetylation to amides, phenylcarbamates, or (xv) synthesis of
 10 Mannich bases, imines, or transformation of ketones or aldehydes to Schiff's bases, oximes, acetals, ketals, enolesters, oxazolidines, thiazolidines or combinations thereof.

The present invention also relates to a nucleic acid molecule encoding a (poly)peptide comprising the sequence

- 15 (a) Met – Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser – Gly – Gly – Gly – Ser (SEQ ID NO: 1);
- (b) Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser – Gly – Gly – Gly – Ser (SEQ ID NO: 2);
- (c) Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser (SEQ ID
 20 NO: 3);
- (d) Ser – Gly – Gly – Ala – Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser – Gly – Gly – Gly – Ser (SEQ ID NO: 4);
- (e) Ser – Gly – Gly – Ala – Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Ala – Ser – Gly – Gly – Gly – Ser (SEQ ID NO: 5);
- 25 (f) Ser – Gly – Gly – Ala – Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ala – Gly – Gly – Gly – Ser (SEQ ID NO: 6);
- (g) Ser – Gly – Gly – Ala – Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser – Gly – Arg – Gly – Ser (SEQ ID NO: 7);
- (h) Ser – Gly – Gly – Ala – Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala
 30 – Pro – Ser – Asp – Gly – Gly – Leu (SEQ ID NO: 8);

- 5 (i) Ser – Gly – Gly – Ala - Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala
– Pro – Ser – Gly – Glu – Gly – Ser (SEQ ID NO: 9);
- (j) Ser – Gly – Gly – Ala - Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala
– Pro – Ser – Gly – Gly – Gly – Trp (SEQ ID NO: 10);
- (k) Ser – Gly – Gly – Ala - Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala
10 – Pro – Ser – Gly – Gly – Cys – Ser (SEQ ID NO: 11);
- (l) Ser – Gly – Gly – Ala - Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala
– Pro – Ser – Gly – Gly – Asp – Ser (SEQ ID NO: 12);
- (m) Ser – Gly – Gly – Ala - Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala
– Pro – Ser – Gly – Gly – Arg – Ser (SEQ ID NO: 13);
- 15 (n) Ser – Gly – Gly – Ala - Trp – Thr – Trp – Asn – Ala – Phe – Ala – Phe – Ala – Ala
– Pro – Ser – Gly – Gly – Gly – Ser (SEQ ID NO: 14);
- (o) Ala – Val – Ser – Tyr – Thr - His – Leu – Gly – Gly – Ala – Gly – Gly – Ala – Trp
– Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser – Gly – Gly – Gly
– Ser (SEQ ID NO: 15);
- 20 (p) Ala – Val – Ser – Tyr – Thr - His – Leu – Ser – Gly – Gly – Ala – Gly – Gly – Ala –
Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser – Gly – Gly –
Gly – Ser (SEQ ID NO: 16);
- (q) Leu – Ser – Leu – Ile – His – Ile – Ser – Gly – Gly – Ala – Ser – Gly – Gly – Ala –
Trp – Thr – Trp – Asn – Ala – Tyr – Ala – Phe – Ala – Ala – Pro – Ser – Gly – Gly –
25 Gly – Ser (SEQ ID NO: 17);

or a nucleic acid molecule, the complementary strand of which hybridizes under stringent conditions to the nucleic acid molecule of any one of (a) to (q), wherein said nucleic acid molecule encodes an interaction partner which is capable of modulating the activity of a nucleic acid binding protein.

- 30 The term “hybridizes under stringent conditions”, as used in the description of the present invention, is well known to the skilled artisan and corresponds to conditions of high stringency. Appropriate stringent hybridization conditions for each nucleic

5 acid sequence may be established by a person skilled in the art on well-known parameters such as temperature, composition of the nucleic acid molecules, salt conditions etc.; see, for example, Sambrook et al., "Molecular Cloning, A Laboratory Manual"; CSH Press, Cold Spring Harbor, 1989 or Higgins and Hames (eds.), "Nucleic acid hybridization, a practical approach", IRL Press, Oxford 1985, see in
10 particular the chapter "Hybridization Strategy" by Britten & Davidson, 3 to 15. Stringent hybridization conditions are, for example, conditions comprising overnight incubation at 42° C in a solution comprising: 50% formamide, 5x SSC (750 mM NaCl, 75 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/ml denatured, sheared salmon
15 sperm DNA, followed by washing the filters in 0.1x SSC at about 65°. Other stringent hybridization conditions are for example 0.2 x SSC (0.03 M NaCl, 0.003M sodium citrate, pH 7) bei 65°C. In addition, to achieve even higher stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). Note that variations in the above
20 conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include, but are not limited to, Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may
25 require modification of the hybridization conditions described above, due to problems with compatibility. Also contemplated are nucleic acid molecules encoding an interaction partner of a biomolecule, wherein the interaction partner is capable of modulating the activity said biomolecule and wherein the nucleic acid molecules hybridize to the nucleic acid molecule encoding the biomolecule at even lower
30 stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are, for example, accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37 degree C in a solution comprising
35 6X SSPE (20X SSPE = 3M NaCl; 0.2M NaH₂PO₄; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA; followed by washes at 50 degree C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower

5 stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). Note that variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include, but are not limited to, Denhardt's
10 reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.

Preferably, said nucleic acid molecule hybridizing to the nucleic acid molecule
15 encoding the interaction partner of a biomolecule has a sequence identity of at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 99.5% when compared to the nucleic acid molecule encoding the interaction partner. The term "which is at least 80% identical" as used in the present invention, relates to sequence identity as determined by the Bestfit® program (Wisconsin Sequence
20 Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit® uses the local homology algorithm of Smith and Waterman to find the best segment of homology between two sequences (*Advances in Applied Mathematics* 2:482-489 (1981)). When using Bestfit® or any other sequence alignment program to determine
25 whether a particular sequence is, for instance, 80% identical to a reference sequence, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide or amino acid sequence and that gaps in homology of up to 5% of the total number of nucleotides or amino acids in the reference sequence are allowed. The identity between a first sequence
30 and a second sequence, also referred to as a global sequence alignment, may also be determined by using the FASTDB computer program based on the algorithm of Brutlag and colleagues (*Comp. App. Biosci.* 6:237-245 (1990)). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of said global
35 sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary,

5 k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter.

The present invention also relates to a (poly)peptide encoded by the nucleic acid of the present invention. Preferably, the interaction partner of the present invention
 10 contains one copy of the peptide sequence encoded by the nucleic acid molecule of the present invention. However, also encompassed by the present invention are (poly)peptides which contain two, three, four, five or more copies of the peptide sequence encoded by the nucleic acid molecules of the present invention or of any interaction partner as defined in the present invention. Also encompassed by the
 15 present invention are (poly)peptides with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 99.5% amino acid sequence identity to the (poly)peptides encoded by the nucleic acid molecules of the present invention, wherein these (poly)peptides are capable of interacting with a biomolecule and of modulating its function.

20 The present invention also relates to an expression vector, comprising an expression control sequence, a multiple cloning site for inserting a gene of interest and the nucleic acid molecule of the present invention, wherein the gene of interest is inserted in-frame with the ORF encoding the peptide. The term "ORF" means "open reading frame" and relates to a nucleotide sequence encoding and capable of
 25 expressing a (poly)peptide.

The present invention also relates to a vector comprising the nucleic acid molecule of the present invention. Preferably said vector is a transfer or expression vector selected from the group consisting of pACT2; pAS2-1; pBTM116; pBTM117; pcDNA3.1; pcDNA1; pECFP; pECFP-C1; pECFP-N1; pECFP-N2; pECFP-N3;
 30 pEYFP-C1; pFLAG-CMV-5 a, b, c; pGAD10; pGAD424; pGAD425; pGAD427; pGAD428; pGBT9; pGEX-3X1; pGEX-5X1; pGEX-6P1; pGFP; pQE30; pQE30N; pQE30-NST; pQE31; pQE31N; pQE32; pQE32N; pQE60; pSE111; pSG5; pTK-Hyg; pTL1; pTL10; pTL-HA0; pTL-HA1; pTL-HA2; pTL-HA3; pBTM118c; pGEX-6P3; pACGHLT-C; pACGHLT-A; pACGHLT-B; pUP; pcDNA3.1-V5His; pMalc2x;. Said
 35 expression vectors may particularly be BAC (bacterial artificial chromosome) and

5 YAC (yeast artificial chromosome) plasmids, cosmids, viruses, bacteriophages and
transposable genetic elements used conventionally in genetic engineering that
comprise the aforementioned nucleic acid. Preferably, said vector is a gene transfer
or targeting vector. Expression vectors derived from viruses such as retroviruses,
vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, or bovine
10 papilloma virus, may be used for delivery of the nucleic acid into targeted cell
population. Methods which are well known to those skilled in the art can be used to
construct recombinant viral vectors; see, for example, the techniques described in
Sambrook et al., *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor
Laboratory (1989) N.Y. and Ausubel et al., *Current Protocols in Molecular Biology*,
15 Green Publishing Associates and Wiley Interscience, N.Y. (1989).

In yet a further preferred embodiment of the invention the vector contains an
additional expression cassette for a reporter protein, selected from the group
consisting of β -galactosidase, luciferase, green fluorescent protein and variants
thereof.

20 Preferably, said vector comprises regulatory elements for expression of said nucleic
acid molecule. Accordingly, the nucleic acid of the invention may be operatively
linked to expression control sequences allowing expression in eukaryotic cells.
Expression of said nucleic acid molecule comprises transcription of the sequence of
the nucleic acid molecule into a translatable mRNA. Regulatory elements ensuring
25 expression in eukaryotic cells, preferably mammalian cells, are well known to those
skilled in the art. They usually comprise regulatory sequences ensuring initiation of
transcription and, optionally, a poly-A signal ensuring termination of transcription
and stabilization of the transcript, and/or an intron further enhancing expression of
said nucleic acid. Additional regulatory elements may include transcriptional as well
30 as translational enhancers, and/or naturally-associated or heterologous promoter
regions. Possible regulatory elements permitting expression in eukaryotic host cells
are the AOX1, CYC1, or GAL1 promoter in yeast or the CMV-, SV40-, RSV-
promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron
in mammalian and other animal cells. Beside elements which are responsible for the
35 initiation of transcription such regulatory elements may also comprise transcription
termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream

5 of the nucleic acid molecule. Furthermore, depending on the expression system used, leader sequences capable of directing the (poly)peptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the aforementioned nucleic acid and are well known in the art. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and
10 termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an C- or N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant
15 product. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDVI (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3, the EchoTM Cloning System (Invitrogen), pSPORT1 (GIBCO BRL) or pCI (Promega).

The present invention also relates to a host cell containing the nucleic acid molecule
20 of the present invention or the expression vector of the present invention. Preferably, the host cell is a eukaryotic or prokaryotic cell. The cell can be the cell of a uni-cellular or multi-cellular organism, which may be pathogenic or non-pathogenic. Preferably, the host cell is selected from mammalian, insect, nematode, plant, yeast, protist cell, Gram-positive or Gram-negative bacteria, archaebacteria or
25 protozoa.

In a preferred embodiment, the nucleic acid molecule of the present invention is fused in frame to at least one chromosomal sequence encoding a (poly)peptide. In another preferred embodiment, the host cell of the present invention also contains (a) the coding sequence of a reporter protein which is under control of a nucleic acid
30 binding protein; and (b) the coding sequence of the nucleic acid binding protein of (a), wherein said coding sequences are operatively linked to expression control sequences. As a consequence of the fusion of the nucleic acid molecule of the present invention to the chromosomal sequence encoding a (poly)peptide, said (poly)peptide is capable of binding to the nucleic acid binding protein which will
35 result in a modulation of gene expression of the reporter protein controlled by said nucleic acid binding protein. In cases when the nucleic acid binding protein is a

- 5 repressor, the negative or repressing effect will be suspended so that gene expression can start or proceed. In cases when the nucleic acid binding protein is an enhancer or activator, binding of the fusion protein to the nucleic acid binding protein will result in activation of gene expression and in production of the reporter gene product or protein.
- 10 In a preferred embodiment of the present invention's host cell, the nucleic acid binding protein is a repressor of transcription. In another preferred embodiment of the present invention's host cell, the nucleic acid binding protein is an enhancer of transcription. Preferably said enhancer of transcription is cAMP responsive protein (CRP) or AraC.
- 15 In a particularly preferred embodiment of the present invention, said nucleic acid binding protein is a (poly)peptide comprising the (poly)peptide sequence of (a) Tet repressor; (b) Lac repressor; (c) Xylose repressor, (d) AraC protein; (e) TetR-based T-Rex system; (f) erythromycin-specific repressor MphR(A); (g) Pip (pristinamycin interacting protein); (h) ScbR from *Streptomyces coelicolor*; (i) TraR of
- 20 *Agrobacterium tumefaciens*; fused to the eukaryotic activation domain p65 of NF κ B; or (j) chimeric proteins. Preferably said chimeric proteins consist of (i) Gal4 DNA-binding domain and either (1) a full-length PhyA protein (PhyA-GBD) or (2) the N-terminal photosensory domain of PhyB [PhyB(NT)-GBD] of *Arabidopsis thaliana*; (ii) steroid hormone regulated systems like the GeneSwitch regulatory system from
- 25 Valantis (www.geneswitch.com), sold by Invitrogen (catalog number K1060-02) and consisting of (1) a Gal4 DNA-binding domain fused to a human progesterone receptor ligand binding domain and an NF κ B-derived p65 eukaryotic transcription activation domain and (2) the inducer mifepristone; (iii) Dimerizer systems from ARIAD consisting of (1) a ZFHD1 DNA-binding domain fused to FKBP 12, (2) a
- 30 modified form of FRAP, termed FRB, fused to the NF κ B-derived p65 activation domain and (3) rapamycin, AP22565 or AP12967 as heterodimer-forming agents, as they bind to both (1) and (2).or (iv) Ecdysone-Inducible Expression System (with the Inducing Agents Ponasterone A and Muristerone A) from Invitrogen (catalog numbers K1001-01 K1003-01, K1004-01) containing (1) a modified form of the
- 35 *Drosophila* ecdysone receptor (VgEcR) fused to a VP16 activation domain, (2) the mammalian homologue RXR of *ultraspiracle*, the natural binding partner of the

5 ecdysone receptor in *Drosophila* and (3) the inducers ponasterone A and muristerone A.

The present invention also relates to an ensemble of host cells of the present invention, wherein said ensemble comprises two or more cells, each of which contain at least one nucleic acid molecule fused in frame to an open reading frame
10 encoding a (poly)peptide. The term "ensemble of host cells" refers to a population of cells, wherein said population contains cells with differently tagged host proteins. Preferably, an individual cell only contains one open reading frame tagged with nucleic acid sequences encoding the interaction partner of the regulatory biomolecule. However, in some cases it may be desirable that 2, 3, 4, 5, 6, 7, 8, 9,
15 or more ORFs contain nucleic acid sequences encoding the interaction partner of the regulatory biomolecule. Preferably, the ensemble or population of cells in their sum are characterized by representing the entire proteome of the host.

In a preferred embodiment of the present invention's host cell, the nucleic acid binding protein is an enhancer or activator of transcription.

20 In another preferred embodiment, the ensemble of host cells of the present invention contains subpopulations with different open reading frames being fused to said nucleic acid molecule of the present invention. In a more preferred embodiment of the present invention the sum of said open reading frames forms the proteome of the host cell.

25 The present invention also relates to a non-human animal containing the host cell of the present invention or the ensemble of host cells of the present invention. The term "non-human" relates to vertebrate and invertebrate animals. Preferred animals are *Drosophila*, mouse, rat, rabbit, monkey and cat. Hosts for pathogens can be inoculated intravenously with pathogens (Ji et al., 1999; Nakayama et al., 1998), by
30 intraperitoneal injection (van Deursen et al., 2001), subcutaneous injection (Dubey et al., 2001), injection into the abdomen (Dionne et al., 2003), aerosol infection by inhalation (Schwebach et al., 2002), by infection with natural hosts (van Deursen et al., 2001), or by simple feeding (Dubey et al., 2001).

- 5 The present invention also relates to a kit comprising (a) the nucleic acid molecule, (b) the (poly)peptide, (c) the vector and/or (d) the host cell or ensemble of host cells of the present invention and (e) instructions for use; in one or more containers.

Finally, the present invention relates to the use of the (poly)peptide of the present invention, the nucleic acid molecule of the present invention, the expression vector
10 of the present invention or the host cell or ensemble of host cells of the present invention for monitoring expression of a gene.

5 Figures:

Figure 1: Experimental procedure for the *in vitro* selection. For the phage display (Kay et al., 2001) experiments, an M13 phage bank (Mourez et al., 2001) of about 10^9 different dodecapeptides was used. After the first round of selection, binding phages were eluted unspecifically by low pH. In round 2, the eluate from round 1 was split into 3 different modes of elution, which were also used in round 3. The output phage titer was determined after each round (shown as pfu/ml for TetR-specific elution). The $\approx 400x$ increase indicates the enrichment of binding phages. After the third round individual clones were isolated, sequenced and characterised for TetR-binding by ELISA.

Figure 2: Example for *in vitro* selected sequences. The first five candidates were obtained by unspecific elution using a glycine-containing buffer at pH2.2. The second five, containing the peptide pepBs1, were obtained by specific elution with $4\mu\text{M}$ TetR(B).

Figure 3: Characterisation of TetR-phage binding by ELISA. To test for TetR-phage-interactions (Kay et al., 2001), 96-well microtiter plates were coated with TetR(B) or bovine serum albumin (BSA) as negative control. M13-phage clones were then added in increasing concentrations. After incubation and several washing steps, a monoclonal Anti-M13 antibody coupled to horseradish peroxidase was added. TetR-M13 interaction was detected by dye formation, after adding the substrate ABTS (2',2'-Azino-bis(ethylbenzthiazolin-6-sulfonic acid). The abbreviations (s) and (us) represent specific and unspecific elution, respectively, pfu represents "plaque forming units".

Figure 4: Design of the peptide expressing construct. The *E. coli* protein thioredoxin A (TrxA) is a small and highly soluble cytoplasmic protein which serves as the carrier (Park and Raines, 2000) for the *in vitro* selected peptides. The *trxA* gene was cloned under the expression control of P_{tac} (Ettner et al., 1996). The *in vitro* selected peptides were fused with a spacer to the solvent exposed C-terminus of TrxA. They also contained the M13 linker present in the initial selection. The expression of the TrxA-peptide fusion proteins is induced by addition of IPTG.

5 **Figure 5: Setup of the *in vivo* screening system.** The screening was carried out in an *E. coli* strain containing the *lacZ* gene under Tet control (Smith and Bertrand, 1988). pWH510/*lacI*^q expresses TetR constitutively (Altschmied et al., 1988). TetR binds to its operator sequence and thereby prevents transcription of the reporter gene. pWH610*trxA-pepX* encodes a fusion protein (*pepX* stands for any *in vitro* selected peptide). Its expression is induced by addition of IPTG (Ettner et al., 1996).
 10 If a peptide fused to thioredoxin binds and induces TetR, β -galactosidase is expressed. This can be detected on MacConkey plates and quantified in LacZ assays.

Figure 6: MacConkey plate. The *E. coli* strain DH5 α (λ *tet50*) was transformed with
 15 plasmids shown in the table above and streaked out to single colonies on MacConkey plates (+ 30 μ M IPTG).

Figure 7: LacZ assay for the TetR-inducing fusion protein TrxA-pepBs1. This assay was carried out using the *in vivo* system from figure 6. The repressed state is symbolized with light grey bars, the tc-induced state with dark grey bars. TrxA without the selected peptide was used as a negative control. Upon induction with
 20 125 μ M IPTG, the *in vitro* selected peptide pepBs1 (as fusion to TrxA) induces TetR(B), which was used as target during the selection. In contrast, a TetR(BD) chimera, in which the protein core containing the inducer-binding and dimerisation domains of TetR(B) (shown in light grey in the cartoon) is replaced by the sequence
 25 of TetR from class D (shown in dark grey in the cartoon) is not induced. The single chain TetR (scTetR) (Krueger et al., 2003) of class B, in which the C-terminus of one monomer is linked to the N-terminus of the other monomer, is also induced.

Figure 8: Identification of the region of interaction between TetR and TrxA-pepBs1 by *in vivo* epitope mapping. To delimit the region of interaction, *in vivo* epitope mapping was carried out. For that purpose, we constructed a set of 17
 30 TetR(BD) chimeras (Schnappinger et al., 1998), in which different parts of α 4 to α 10 were replaced by the TetR(D) sequence, and characterised them for inducibility *in vivo*. We exploited the fact that TetR(B) is inducible by the peptide, but TetR(BD) is not – a B-D exchange which leads to a loss of induction thus indicates the
 35 importance of at least part of the replaced region. The *in vivo* inducibility of a

5 chimera is marked with + or -. Characterisation of these chimeras resulted in a defined region of interaction spanning from the beginning of $\alpha 8$ up to the first amino acids of $\alpha 10$.

Figure 9: Structure of TetR. One monomer is shown in black, the other in dark grey. Mg^{2+} -Tetracyclines are shown as white stick models. A class B to D exchange
10 in the portion depicted in light grey leads to a loss of inducibility and represents the region involved in induction by the peptide pepBs1.

Figure 10: Expression of the peptide correlates with induction of TetR. For this experiment, cells were grown under uninduced and induced conditions using IPTG concentrations between 1 μM and 500 μM . The viability was determined by OD_{600}
15 measurement and crude lysates were prepared to detect the expression level of the fusion protein. LacZ measurements were carried out to determine the induction of TetR.

A. Expression of the peptide fusion. Lane 2 of the Western blot shows TrxA uninduced, lane 3 expressed TrxA after induction with 500 μM IPTG. Lane 4 to 12
20 show the expression of the fusion protein TrxA-pepBs1 using increasing IPTG concentrations (see table). **B. Induction of TetR** (LacZ data compared to OD_{600} and [IPTG]). The β -galactosidase activity is depicted as a black curve. TetR induction is first observed using 15 μM IPTG to induce (marked with a black arrow) the expression of TrxA-pepBs1. Maximum induction was reached at an IPTG
25 concentration of 60 μM . Up to that point, the expression level of TrxA-pepBs1 correlates with the induction of TetR. Higher concentrations of the fusion protein appear to have a negative effect on cell viability, as can be seen by the decrease of the OD_{600} (see grey curve). Concomitantly, the steady state level of the fusion protein is diminished and the induction of TetR is also reduced.

30 **Figure 11: *In vivo* characterisation of non-inducible TetR mutants.** The observation that all TetR(BD) chimeras shown in figure 9 are inducible by tc, but only a few are inducible with the peptide, provided the first indication that induction of TetR by the peptide is mechanistically different from induction by tc. To substantiate this assumption we analyzed TetR mutants (Müller et al., 1995) which

5 carry a single amino acid exchange in tc-contacting residues *in vivo*. The repression of the reporter gene by TetR is shown with light grey bars, the induction with tc with dark grey bars. The mutants H64Y, N82A and F86A were not or only slightly inducible with tetracycline but either fully or at least partially inducible with the peptide (see black bars).

10 **Figure 12: Position of the amino acids H64, N82 and F86 relative to tetracycline and the interaction epitope.** The region of TetR-peptide interaction mapped for each monomer is shown in light and dark grey, respectively. Tc is depicted as light grey stick model. The residues in the mutants which showed the phenotype of being inducible by the peptide but not by tc, when exchanged, are
15 grouped around the A-ring of tc.

Figure 13: Amino acids contacting tc either directly or indirectly via the hydrated magnesium cation (tc is shown as light grey stick model).

Figure 14: *In vivo* characterisation of TetR inducibility by TrxA fusion proteins.

A. β -Galactosidase measurement. This assay is carried out in *E. coli* DH5 α (λ tet50) which carries the *lacZ* gene under Tet-control as a single copy integrated into the *E. coli* genome. The first bar shows the repressed state when TetR binds to its operator sequence *tetO*. After addition of tetracycline, TetR is induced and β -galactosidase is expressed. TrxA fusion proteins are under transcription control of P_{tac} and induced by addition of IPTG. TrxA without the fused peptide was used as a
20 control and is not capable of inducing TetR. After addition of 60 μ M IPTG, the C-terminal TrxA-peptide fusion induces TetR about 14-fold. The N-terminal fusion reaches a factor of induction of about 40 and is, thus, more effective.
25

B. Western Blot analysis. A monoclonal TrxA antibody was used to detect TrxA and TrxA fusion proteins in samples which were used simultaneously for carrying out β -galactosidase activity measurements and for preparing crude lysates. The data
30 indicates that all proteins were expressed at the same level and the increase of induction seen for the N-terminal fusion is not due to a higher protein amount.

5 **Figure 15: Correlation between the protein level of PepBs1-TrxA and induction of TetR(B).**

β -galactosidase measurement and Western blot analysis were carried out as explained in figure 14. The N-terminal TrxA-peptide fusion was induced using IPTG concentrations ranging from 1 μ M up to 125 μ M. The Western blot data shows that
10 the amount of fusion protein correlates to the induction of TetR(B) as reflected in the increase in β -galactosidase activity. A maximum of induction was reached using 15 μ M IPTG.

15 **Figure 16: Comparison of constitutive expression systems leading to low and medium steady-state levels of TetR and their influence on the induction profile by PepBs1-TrxA.**

β -Galactosidase measurements were carried out as detailed in figures 14 and 15 with the exception of TetR(B) being expressed from different plasmids. pWH510/*acI*^R expresses TetR constitutively at a low level (shown in dark grey bars) whereas pWH1411/*acI*^R expresses TetR constitutively at a medium level (shown in light grey
20 bars). In the low expressing system, induction of TetR is observed even for very low IPTG concentrations which is equivalent to a low protein level of the TrxA-peptide fusion (see figure 15). The maximum β -galactosidase activity is reached at IPTG concentrations of 15 μ M or higher. The higher amount of TetR in the medium expression system leads to a delayed increase in β -galactosidase activity requiring
25 the presence of higher amounts of the fusion protein for full induction. Consequently, the maximum of β -galactosidase activity is reached at 60 μ M IPTG and higher.

Figure 17: Comparison of TetR(B) induction by C- and N-terminal TrxA-peptide fusions.

30 β -galactosidase measurements were carried out as explained in figure 14 and using the low-level TetR-expression system pWH510/*acI*^R. While induction of TetR by the C-terminal fusion is characterised by a curve having a weakly ascending slope, induction of TetR by the N-terminal fusion is characterised by a curve profile with a

- 5 steeply ascending slope. In addition, the N-terminal fusion leads to an about 3-fold higher induction compared to the C-terminal fusion.

Figure 18: LacZ assay for the TetR-inducing fusion protein SbmC-pepBs1.

The sbmC (gyrI) gene was cloned under the expression control of Ptac into a pWH610 vector background (see Figure 5). The peptide pepBs1 containing the M13 linker present in the initial selection was fused with a spacer to the solvent-exposed C-terminus or N-terminus of SbmC. The expression of the SbmC-peptide fusion proteins is induced by addition of IPTG. The β -galactosidase activity measurement was carried out as described in Figure 14. TrxA and SbmC without the fused peptide were used as controls and do not induce the more sensitive variant
10 TetR(B/D)188-208 encoded by a pWH510lacIq background. After addition of 60 μ M IPTG, all tagged constructs induce β -galactosidase expression. Most active are both N-terminal fusion proteins, followed by the TrxA(C)-pepBs1 fusion. The least active inducer is the SbmC(C)-pepBs1 fusion. But even it leads to a 4-fold increased β -galactosidase activity.
15

20 **Figure 19: An in-frame fusion of an insertion element (IEFSK) encoding pepBs1 to TrxA induces TetR(B).**

A. Genetic organization of an in-frame fusion of the transposable sequence element IEFSK-pepBs1 to TrxA. The trxA gene is displayed as a grey, the kanamycin resistance gene as a white arrow. Their cognate promoters are shown at the respective positions as small black arrows. The insertion element IEFSK-pepBs1 is bordered by ME elements which are displayed as black triangles. The flexible linker connecting the ME element with pepBs1 is indicated by a hatched box and its length, pepBs1 by a grey box. The lollipop denotes a Rho-independent transcriptional terminator and the FRT sites flanking the resistance cassette are shown. The reading frame formed by the TrxA-pepBs1 fusion is displayed above the respective genetic elements and the position of the stop codon terminating translation is marked by an "X". The seven amino acids encoded by the ME element in the fusion protein are detailed above the element.
25
30

B. LacZ assay for the TetR-inducing fusion protein TrxA-pepBs1 derived from IEFSK-pepBs1. The transposable sequence element IEFSK-pepBs1 was cloned downstream of the TrxA gene yielding a fusion protein TrxA-pepBs1. It is under
35

- 5 expression control of Ptac in a pWH610 vector background (see Figure 5) The expression of the TrxA-peptide fusion protein is induced by addition of IPTG. The β -galactosidase activity measurement was carried out as described in Figure 14. After addition of increasing amounts of IPTG, both TrxA fusion constructs (wt TrxA-pepBs1 from pWH2101 and ME-linker containing TrxA-pepBs1 from pWH1909)
- 10 induce β -galactosidase expression identically in a concentration-dependent manner. This indicates the the additional sequence introduces by the ME element do not interfere with the TetR(B) inducing properties of the Bs1 peptide.

Figure 20: An in-frame fusion of the insertion element IEFSK containing pepBs1 to the atpD ORF at its endogenous location in the E. coli genome

15 **leads to a protein that induces TetR(B).**

- The atpD gene from Escherichia coli is shown schematically with its length in base-pairs indicated. The nucleotide position of the IEFSK-pepBs1 element's insertion site and its orientation are given above the gene. Black triangles correspond to the mosaic element inverted repeats, the inducing peptide is indicated by a white box
- 20 and KmR denotes a kanamycin-resistance expression cassette. The sequencing reaction of the genomic DNA from E. coli IEFSK0001 detailing the exact insertion site is shown for the 3' end of the insertion element. At right, a MacConkey agar plate is shown with streak-outs of the parental strain WH207(λ tet50)/pWH1911 (left half) which does not express β -galactosidase and the tagged strain IEFSK0001
- 25 containing the tagged atpD gene (right half) which expresses β -galactosidase.

- 5 The examples illustrate the invention

Example 1: *In vitro* selection of TetR-binding peptides

Several different experimental approaches can be employed to identify novel ligands for proteins. The screening of low molecular weight compound libraries permits the identification of small molecules that bind to a given target protein.

- 10 These libraries can consist of a diverse collection of natural products or, alternatively, contain a large set of synthetic compounds generated by combinatorial chemistry. A completely different approach is the use of *in vitro* evolution methods (Lin and Cornish, 2002). These allow the selection of biological macromolecules that display affinity to a defined target molecule. The SELEX method (Systematic
15 Evolution of Ligands by EXponential Enrichment) allows the isolation of nucleic acids, RNA or DNA, that bind a defined target with high affinity and specificity (Tuerk and Gold, 1990). Several methods have been developed to select peptide ligands. Two more recent developments are "mRNA-display" or "ribosome display" (Wilson et al., 2001). A more common method is "phage display" (Giannattasio and
20 Weisblum, 2000) which was also used in our selection for TetR-binding peptides.

- Phage display: The basis for most phage display applications is the filamentous phage M13 from *Escherichia coli*. A surface protein, gpIII, which is present in five copies per phage and important for infection of the bacteria can tolerate N-terminal insertions to a certain degree. Since the N-terminus of this protein is solvent-
25 exposed, the insertions are presented on the surface of the phage (Kay et al., 2001). The commercially available phage bank Ph.D.-12TM from New England Biolabs was used to select for TetR binding peptides. It contains $\sim 10^9$ different dodecapeptides fused via a flexible linker of four amino acids to the N-terminus of the gpIII protein. The *in vitro* selection was performed by coating polystyrene tubes
30 (NUNC Maxisorb) with purified Tet repressor protein from class B [TetR(B) (Ettner et al., 1996)]. The tubes were then incubated with the pool of M13 phages, washed several times and TetR(B)-bound phages were eluted either specifically by addition of TetR(B) or unspecifically by lowering the pH value. Three selection cycles were performed and the enrichment of TetR-binding phages was monitored by

- 5 determining the M13 titer after each round (Figure 1). Individual M13 clones were picked and sequenced after the third selection round (Figure 2).

Immunological detection of the M13-TetR-interaction: Binding of individual M13 clones to TetR was determined by ELISA (Enzyme Linked Immunosorbent Assay). The phages were amplified, precipitated and resuspended in a small volume. 96-
 10 well microtiter-plates were coated with TetR(B), then blocked with Bovine Serum Albumin, followed by incubation with increasing amounts of M13 phages from different isolates, several washes with buffer and, finally, incubated with an M13-specific monoclonal antibody covalently coupled to horseradish peroxidase. Addition
 15 of ABTS (2', 2'-Azino-bis(ethylbenzthiazolin-6-sulfonic acid) as substrate permits the spectrophotometric detection of phage-binding to TetR. The degree of absorption serves as a quantitative indicator of phages bound to the target protein (Kay et al., 2001). For the peptide pepBs1 which will be discussed in more detail in the following sections, the A_{TetR}/A_{BSA} factor determined with the phage-dilution containing 10^{10} pfu was 34 (Figure 3).

20 **Example 2: *In vivo* screening for TetR-inducing peptides**

Establishing an *in vivo* screen in *Escherichia coli*: After having obtained and identified several TetR-binding peptides, the next goal was to isolate peptides that could induce TetR(B). Since small oligopeptides are rapidly degraded intracellularly by proteases, the peptide-encoding sequences were cloned as C-terminal fusions to
 25 the *Escherichia coli* protein thioredoxin, an established carrier protein for peptides (Park and Raines, 2000). The thioredoxin fusion proteins were expressed by a *tac* promoter under control of Lac repressor and, thus, inducible by addition of IPTG (Isopropyl- β -thiogalactoside) (Ettner et al., 1996) (see Figure 4 for illustration). TetR(B) was expressed constitutively at a low level (Altschmied et al., 1988). The
 30 indicator strain was *E. coli* DH5 α ($\lambda tet50$) containing the phage $\lambda tet50$ (Smith and Bertrand, 1988) integrated in single copy into the *E. coli* genome. This phage contains a *tetA-lacZ* transcriptional fusion. Expression of β -galactosidase is, thus, regulated by TetR that binds to *tetO* sequences located within the promoter (Figure 5). The pool of TetR-binding peptides is screened by plating transformed colonies
 35 on MacConkey agar containing IPTG. An inducing Trx-peptide fusion protein will

- 5 lead to the expression of β -galactosidase, resulting in an acidification of the medium surrounding the colony which can be detected by its yellow color (Figure 6, sectors 1, 2, 4, and Figure 20, right half).

Cloning of individual candidates: Individual candidates identified by sequencing were cloned as C-terminal fusions to TrxA, introduced into the reporter strain
 10 DH5 α (λ tet50) and the respective β -galactosidase activities determined. The following controls were also included in the measurements: To define the regulatory window, both the repressed state (0% - TetR binds to *tetO*) and the fully induced state in the presence of tc (100% - TetR dissociates from *tetO*) were determined. To
 15 exclude that thioredoxin is involved in the TetR-peptide interaction, a plasmid expressing thioredoxin without a peptide fusion was also assayed in the presence and absence of IPTG. The β -galactosidase activities of the individual candidates cloned were also determined in the presence and absence of IPTG.

β -Galactosidase activity assays: A TetR-inducing Thioredoxin-peptide fusion protein was identified in the pool of cloned TetR-binding peptides. The fusion protein is
 20 specific for TetR(B), since a chimeric repressor, TetR(B/D), is not induced in the presence of Trx-pepBs1. This chimera consists of the DNA-binding domain (residues 1–50) from TetR(B) and the protein core with the inducer-binding and dimerization domains from TetR(D). The sequence identity between the two classes is 63 % at the amino acid level. A single-chain TetR(B) variant in which both
 25 subunits of TetR are fused to a monomer by a flexible protein linker connecting the C-terminus of one subunit to the N-terminus of the other was also induced by TrxA-pepBs1 (Figure 7). This suggests than an interaction of the peptide with the dimerization surface of TetR and subsequent induction of TetR by dissociation appear unlikely. The sequence of the peptide pepBs1, with linker elements, is
 30 shown in Figure 4.

Example 3: Identification of the interaction site between TetR and the inducing peptide

We isolated the interaction site of TrxA-pepBs1 with TetR(B) by *in vivo* epitope mapping, taking advantage of the observation that TetR(B/D) is not induced by the

5 peptide. We therefore constructed chimeric repressors in which *tetR*(B) sequences are exchanged to different extents by the corresponding sequences from *tetR*(D) (Schnappinger et al., 1998) and determined their *in vivo* inducibility by TrxA-pepBs1. Figures 8 and 9 summarize the results obtained. The inducibility profile shows that interactions between repressor and peptide are confined to the region from helix α 8
 10 to residue 182 in helix α 10. The loop connecting the helices α 9 and α 10 also appears to be important, as chimeras containing *tetR*(D) sequences at residues 179–184 and 180–184 are not inducible by TrxA-pepBs1.

Example 4: Analysis of TetR mutants with an induction-deficient phenotype

To demonstrate that induction mediated by pepBs1 follows a novel mechanism,
 15 different than that of tetracycline, we cloned induction mutants of TetR(B) (Müller et al., 1995) into the *in vivo* test system. The mutants contain single residue exchanges that lead to an induction-deficient phenotype. The mutant TetR(B)HY64 with an exchange of the histidine residue at position 64 to tyrosine is not inducible by tetracycline. β -Galactosidase activity assays show, however, that it is still
 20 inducible by TrxA-pepBs1 (see Figures 11 to 13 and Table 1).

Example 5: The inducing tag is also active as N-terminal fusion

The versatility of the inducing tag as a marker for protein presence would be greatly enhanced if it were not confined to C-terminal protein fusions, as these need not be active with all proteins (Huh et al., 2003). We therefore fused the inducing peptide
 25 with the M13 linker element genetically to the N-terminus of thioredoxin A from *E. coli*. We expressed this fusion protein from pWH610 in the same *in vivo* assay system as shown in Figure 5. Fusion protein expression was induced by addition of IPTG to a final concentration of 60 μ M, β -galactosidase activities were measured and the fusion protein amounts were determined by Western blotting of crude
 30 extracts. The results shown in Figures 14 and 17 clearly demonstrate that the N-terminal fusion is not only active, but more active than the C-terminal fusion. Its 40-fold induction is higher than the 14-fold induction obtained with the C-terminal fusion. This is not due to higher steady-state amounts of the fusion protein, as is

5 evident from the Western blot data, but rather reflects an intrinsic activity of the protein.

Example 6: The expression level of the tagged protein correlates with reporter gene activity

Quantification of the tagged protein requires a correlation between the measured
 10 reporter gene activity and the amount of the tagged protein expressed. To test if this is the case, we induced expression of the TrxA-peptide fusion protein to different extents by varying the amounts of the inducer IPTG. We then measured the respective β -galactosidase activity and determined the corresponding amounts of the TrxA-peptide fusion protein by Western blotting with a monoclonal antibody
 15 against TrxA. Figures 10 and 15 show that higher amounts of the TrxA-peptide fusion protein lead to higher amounts of β -galactosidase activity. In Figure 15, a plateau is reached at an IPTG concentration of 15 μ M, and β -galactosidase activity is fully induced. This is most likely due to all TetR present in the cell having been bound by the tagged protein. The resolution window can be shifted by increasing the
 20 intracellular steady-state amount of TetR. For example, pWH1411 expresses higher amounts of TetR than pWH510 (Wissmann et al., 1991). Consequently, in the presence of pWH1411, higher amounts of the fusion protein are needed to reach the same β -galactosidase activities as in the presence of pWH510 (compare 2.5 μ M IPTG for pWH510 with 15 μ M IPTG for pWH1411), and the plateau corresponding
 25 to full induction of β -galactosidase activity is only reached at an IPTG concentration of 60 μ M (Figure 16).

Table 1: Repression and inducibility of TetR^S mutants by tc or TrxA-pepBs1.

This table summarises the TetR mutants characterised. They all carry a single amino acid exchange in residues surrounding the inducer tc and are induction-
 30 deficient as TetR(B) or as TetR(D) variants in a different and less sensitive *in vivo* assay system (Müller et al., 1995, Schubert, 2001). The positions of the individual residues relative to tc are shown schematically in Figure 13 with the exception of D53 and E114. Column 2 shows the repressed state in the absence of any inducer, column 3 the tc induced-state. Columns 4 and 5 show the *in vivo* data in the
 35 presence of the TrxA-pepBs1 construct, whereby column 5 represents the peptide-

- 5 induced state after induction with 60 μ M IPTG. Mutants with an at least three fold increase in β -galactosidase activity compared to the repressed state are defined as inducible and are shown in bold. The mutants H64Y, N82A and F86A (shown in detail in Figures 12 and 13) are not or only slightly inducible by tc, but induced with the peptide (Figure 11). The mutants P105A and E114G are inducible by tc, but not
- 10 by the peptide. The same holds true for many of the TetR(B/D) chimeras. The fact that we find no correlation between tc-inducible and peptide-inducible mutants supports the assumption that the mechanisms underlying peptide-induction and tc-induction are different.

pWH510/ <i>lacI^q</i> -derivatives				
TetR(B) variant	β -Galactosidase activity [MU]			
	- tc	+ tc	pWH610/ <i>trxA-pepBs1</i>	
			- IPTG	+ IPTG
wt	142 \pm 7,7	4881 \pm 384	169 \pm 9,0	1996 \pm 56
D53G	157 \pm 6,2	1740 \pm 319	144 \pm 4,4	1322 \pm 86
H64Y	135 \pm 2,9	47 \pm 21	140 \pm 6,2	1212 \pm 83
N82A	165 \pm 3,6	125 \pm 2,4	178 \pm 8,0	618 \pm 8,9
F86A	162 \pm 2,8	480 \pm 33	151 \pm 11	1464 \pm 65
H100Y	125 \pm 11	56 \pm 8,3	129 \pm 9,8	136 \pm 11
T103A	142 \pm 3,6	802 \pm 174	154 \pm 9,9	712 \pm 45
P105A	173 \pm 3,4	4085 \pm 35	185 \pm 9,4	210 \pm 14
E114G	117 \pm 9,8	1574 \pm 196	123 \pm 12	178 \pm 21
E147A	154 \pm 5,8	526 \pm 185	173 \pm 12	629 \pm 77

15 Example 7: The inducing tag is also active when fused to SbmC/Gyrl

- The initial in vivo screen for isolating an inducing peptide was performed by fusing candidate sequences to TrxA. We therefore cannot exclude that sequences from the TrxA carrier protein contribute to or might even be necessary for induction of TetR by pepBs1. To check this, we fused the pepBs1 encoding sequence with a spacer
- 20 region genetically to both N- and C-terminus encoding regions of the sbmC gene from *E. coli*. This gene, also known as gyrl, belongs to the SOS regulon (Oh et al.,

2001) and encodes a protein with solvent-exposed N- and C-termini (Romanowski et al., 2002). We expressed both N- and C-terminal fusion proteins from pWH610-derivatives in the same in vivo assay system shown in Figure 5. Fusion protein expression was induced by addition of IPTG and β -galactosidase activities were determined. The results are shown in Figure 18 and clearly demonstrate that both N- and C-terminal fusions are active in inducing TetR. While the C-terminal fusion of pepBs1 to SbmC is less active than when fused to TrxA, both N-terminal fusions fully induce TetR. As we cannot determine the steady-state protein levels of the SbmC fusion proteins, we cannot distinguish between lower protein levels or impaired intrinsic activity of the SbmC(C)-pepBs1 fusion protein as reason for the reduced activity. But, most important, both fusions to this protein, completely different from TrxA in both sequence and structure, are active in inducing TetR.

Example 8: Expression of an in-frame fusion of the inducing tag to the *atpD* gene in its endogenous genomic context leads to a protein that induces TetR

Up to now, we had only analyzed induction of TetR by plasmid-encoded, conditionally-expressed Bs1-containing fusion proteins (see examples 2, 5, and 7). To determine if in-frame fusion of pepBs1 to a protein expressed from its native genomic context can also lead to induction of TetR, we used transposase-mediated random integration into the genome of *E. coli* WH207(λ tet50)/pWH1911. This approach avoids potential problems due to unknown idiosyncratic effects (expression profile, protein stability, accessibility of the N- and C-terminus) of an individually selected target protein. 40 ng of the IEFSK-Bs1 insertion element (Figure 19) were incubated with a 5-fold molar excess of hyperactive Tn5 transposase (Goryshin and Reznikoff, 1998), used to transform *E. coli* WH207(λ tet50)/pWH1911 and the cells spread out on MacConkey agar plates containing 60 μ g/ml kanamycin to select for integration of the insertion element. The plasmid pWH1911 expresses TetR(B) constitutively at a low level. Three colonies out of 2100 had a yellow phenotype, indicating expression of β -galactosidase. We picked one and re-streaked it until it had a homogenous yellow phenotype on MacConkey agar plates. After verifying that the Tet system components on pWH1911 and λ tet50 had retained wild-type activity, we isolated chromosomal DNA and identified the insertion site by sequencing with two primers. IEFSK-pepBs1 had inserted after nucleotide 1147 of the *atpD* gene leading to its in-frame fusion with pepBs1 (see Figure 20). The *atpD* gene codes for the β -subunit of the F1

- 5 component of ATP synthase. This is the third independent example of induction of TetR by a fusion protein containing the pepBs1 element. The example also shows that expression of a protein from its natural genomic context can be monitored by induction of TetR by the pepBs1 moiety.

5

References

- Altschmied, L., Baumeister, R., Pfeiderer, K. and Hillen, W. (1988). A threonine to alanine exchange at position 40 of Tet repressor alters the recognition of the sixth base pair of *tet* operator from GC to AT. *EMBO J* 7, 4011-4017.
- Aung-Hilbrich, L. M., Seidel, G., Wagner, A., and Hillen, W. (2002). Quantification of
10 the influence of HPrSer46P on CcpA-cre interaction. *J Mol Biol* 319, 77-85.
- Baron, U., Gossen, M. and Bujard, H. (1997). Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res* 25, 2723-2729.
- Breaker, R. R. (2002). Engineered allosteric ribozymes as biosensor components.
15 *Curr Opin Biotechnol* 13, 31-39.
- Calnan, B. J., Biancalana, S., Hudson, D., and Frankel, A. D. (1991). Analysis of arginine-rich peptides from the HIV Tat protein reveals unusual features of RNA-protein recognition. *Genes Dev* 5, 201-210.
- Chan, K., Knaak, T., Satkamp, L., Humbert, O., Falkow, S., and Ramakrishnan, L.
20 (2002). Complex pattern of *Mycobacterium marinum* gene expression during long-term granulomatous infection. *Proc Natl Acad Sci USA* 99, 3920-3925.
- Cherepanov, P. P., and Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158, 9-14.
- Datsenko, K. A., and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97, 6640-6645.
- Deiwick, J., Rappl, C., Stender, S., Jungblut, P. R., and Hensel, M. (2002). Proteomic approaches to *Salmonella* Pathogenicity Island 2 encoded proteins and
30 the SsrAB regulon. *Proteomics* 2, 792-799.

- 5 Dionne, M. S., Ghori, N., and Schneider, D. S. (2003). *Drosophila melanogaster* is a genetically tractable model host for *Mycobacterium marinum*. *Infect Immun* 71, 3540-3550.
- Dubey, J. P., Lindsay, D. S., Kwok, O. C., and Shen, S. K. (2001). The gamma interferon knockout mouse model for sarcocystis neurona: comparison of infectivity
10 of sporocysts and merozoites and routes of inoculation. *J Parasitol* 87, 1171-1173.
- Eichenbaum, Z., Federle, M. J., Marra, D., de Vos, W. M., Kuipers, O. P., Kleerebezem, M., and Scott, J. R. (1998). Use of the lactococcal *nisA* promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. *Appl Environ Microbiol* 64, 2763-2769.
- 15 El Fakhry, Y., Ouellette, M., and Papadopoulou, B. (2002). A proteomic approach to identify developmentally regulated proteins in *Leishmania infantum*. *Proteomics* 2, 1007-1017.
- Epshtein, V., Mironov, A. S., and Nudler, E. (2003). The riboswitch-mediated control of sulfur metabolism in bacteria. *Proc Natl Acad Sci USA* 100, 5052-5056.
- 20 Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J. C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* 47, 103-118.
- Ettner, N., Müller, G., Berens, C., Backes, H., Schnappinger, D., Schreppel, T., Pfeiderer, K., and Hillen, W. (1996). Fast large-scale purification of tetracycline repressor variants from overproducing *Escherichia coli* strains. *J Chromatogr A* 742,
25 95-105.
- Fussenegger, M., Morris, R. P., Fux, C., Rimann, M., von Stockar, B., Thompson, C. J., and Bailey, J. E. (2000). Streptogramin-based gene regulation systems for mammalian cells. *Nat Biotechnol* 18, 1203-1208.
- 30 Geissendörfer, M., and Hillen, W. (1990). Regulated expression of heterologous genes in *Bacillus subtilis* using the Tn10 encoded *tet* regulatory elements. *Appl Microbiol Biotechnol* 33, 657-663.

- 5 Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K. and Weissman, J. S. (2003). Global analysis of protein expression in yeast. *Nature* 425, 737-741.

Glannattasio, R. B. and Weisblum, B. (2000). Modulation of *erm* methyltransferase activity by peptides derived from phage display. *Antimicrob Agents Chemother* 44,
10 1961-1963.

Goryshin, I. Y., Jendrisak, J., Hoffman, L. M., Meis, R., and Reznikoff, W. S. (2000). Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. *Nat Biotechnol* 18, 97-100.

Goryshin, I. Y., and Reznikoff, W. S. (1998). Tn5 *in vitro* transposition. *J Biol Chem*
15 273, 7367-7374.

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 89, 5547-5551.

Gosser, Y., Hermann, T., Majumdar, A., Hu, W., Frederick, R., Jiang, F., Xu, W., and Patel, D. J. (2001). Peptide-triggered conformational switch in HIV-1 RRE RNA
20 complexes. *Nat Struct Biol* 8, 146-150.

Gregory, S. T., Cate, J. H. D., and Dahlberg, A. E. (2001). Streptomycin-resistant and streptomycin-dependent mutants of the extreme thermophile *Thermus thermophilus*. *J Mol Biol* 309, 333-338.

Guina, T., Purvine, S. O., Yi, E. C., Eng, J., Goodlett, D. R., Aebersold, R., and
25 Miller, S. I. (2003). Quantitative proteomic analysis indicates increased synthesis of a quinolone by *Pseudomonas aeruginosa* isolates from cystic fibrosis airways. *Proc Natl Acad Sci USA* 100, 2771-2776.

Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., and Aebersold, R. (2000). Evaluation of two-dimensional gel electrophoresis-based proteome analysis
30 technology. *Proc Natl Acad Sci USA* 97, 9390-9395.

- 5 Hamer, L., DeZwaan, T. M., Montenegro-Chamorro, M. V., Frank, S. A., and Hamer, J. E. (2001). Recent advances in large-scale transposon mutagenesis. *Curr Opin Chem Biol* 5, 67-73.
- Hanash, S. (2003). Disease proteomics. *Nature* 422, 226-232.
- 10 Harada, K., Martin, S. S., and Frankel, A. D. (1996). Selection of RNA-binding peptides *in vivo*. *Nature* 380, 175-179.
- Harada, K., Martin, S. S., and Frankel, A. D. (1999). *In vivo* selection of RNA-binding peptides from combinatorial libraries. *Nucleic Acids Symp Ser*, 213-214.
- Hayes, F. (2003). Transposon-based strategies for microbial functional genomics and proteomics. *Annu Rev Genet* 37, 3-29.
- 15 Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S. and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* 425, 686-691.
- Jain, K. K. (2000). Applications of proteomics in oncology. *Pharmacogenomics* 1, 385-393.
- 20 Ji, Y., Marra, A., Rosenberg, M., and Woodnutt, G. (1999). Regulated antisense RNA eliminates alpha-toxin virulence in *Staphylococcus aureus* infection. *J Bacteriol* 181, 6585-6590.
- Ji, Y., Zhang, B., Van Horn, S. F., Warren, P., Woodnutt, G., Burnham, M. K. R., and Rosenberg, M. (2001). Identification of critical staphylococcal genes using
25 conditional phenotypes generated by antisense RNA. *Science* 293, 2266-2269.
- Judson, N., and Mekalanos, J. J. (2000). Transposon-based approaches to identify essential bacterial genes. *Trends Microbiol* 8, 521-526.
- Kay, B. K., Kasanov, J. and Yamabhai, M. (2001). Screening phage-displayed combinatorial peptide libraries. *Methods* 24, 240-246.

- 5 Koizumi, M., Soukup, G. A., Kerr, J. N., and Breaker, R. R. (1999). Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP. *Nat Struct Biol* 6, 1062-1071.
- Lee, S. J., Boos, W., Bouche, J. P., and Plumbridge, J. (2000). Signal transduction between a membrane-bound transporter, PtsG, and a soluble transcription factor, Mlc, of *Escherichia coli*. *EMBO J* 19, 5353-5361.
- 10 Len, A. C., Cordwell, S. J., Harty, D. W., and Jacques, N. A. (2003). Cellular and extracellular proteome analysis of *Streptococcus mutans* grown in a chemostat. *Proteomics* 3, 627-646.
- Lin, H. and Cornish, V. W. (2002). Screening and selection methods for large-scale analysis of protein function. *Angew Chem Int Ed* 41, 4402-4425.
- 15 Mandal, M., Boese, B., Barrick, J. E., Winkler, W. C., and Breaker, R. R. (2003). Riboswitches control fundamental biochemical pathways in *Bacillus subtilis* and other bacteria. *Cell* 113, 577-586.
- Martzen, M. R., McCraith, S. M., Spinelli, S. L., Torres, F. M., Fields, S., Grayhack, E. J., and Phizicky, E. M. (1999). A biochemical genomics approach for identifying genes by the activity of their products. *Science* 286, 1153-1155.
- 20 Mourez, M., Kane, R. S., Mogridge, J., Metallo, S., Deschatelets, P., Sellmann, B. R., Whitesides, G. M. and Collier, R. J. (2001). Designing a polyvalent inhibitor of anthrax toxin. *Nat Biotechnol* 19, 958-961.
- 25 Müller, G., Hecht, B., Helbl, V., Hinrichs, W., Saenger, W. and Hillen, W. (1995). Characterization of non-inducible Tet repressor mutants suggests conformational changes necessary for induction. *Nat Struct Biol* 2, 693-703.
- Nakayama, H., Izuta, M., Nagahashi, S., Sihta, E. Y., Sato, Y., Yamazaki, T., Arisawa, M., and Kitada, K. (1998). A controllable gene-expression system for the pathogenic fungus *Candida glabrata*. *Microbiology* 144, 2407-2415.
- 30 Neddermann, P., Gargioli, C., Muraglia, E., Sambucini, S., Bonelli, F., De Francesco, R., and Cortese, R. (2003). A novel, inducible, eukaryotic gene

- 5 expression system based on the quorum-sensing transcription factor TraR. EMBO Rep 4, 159-165.

Oh, T. J., Jung, I. L., and Kim, I. G. (2001). The *Escherichia coli* SOS gene *sbmC* is regulated by H-NS and RpoS during the SOS induction and stationary growth phase. Biochem Biophys Res Commun 288, 1052-1058.

- 10 Okinaka, Y., Yang, C. H., Perna, N. T., and Keen, N. T. (2002). Microarray profiling of *Erwinia chrysanthemi* 3937 genes that are regulated during plant infection. Mol Plant Microbe Interact 15, 619-629.

Park, S. H. and Raines, R. T. (2000). Genetic selection for dissociative inhibitors of designated protein-protein interactions. Nat Biotechnol 18, 847-851.

- 15 Peterson, R. D. and Feigon, J. (1996). Structural change in Rev responsive element RNA of HIV-1 on binding Rev peptide. J Mol Bio 264, 863-877.

Phizicky, E., Bastiaens, P. I., Zhu, H., Snyder, M., and Fields, S. (2003). Protein analysis on a proteomic scale. Nature 422, 208-215.

- Romanowski, M. J., Gibney, S. A., and Burley, S. K. (2002). Crystal structure of the
20 *Escherichia coli* SbmC protein that protects cells from the DNA replication inhibitor microcin B17. Proteins 47, 403-407.

- Rosenkrands, I., Slayden, R. A., Crawford, J., Aagaard, C., Barry, C. E., 3rd, and Andersen, P. (2002). Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labeling and proteome analysis of cellular and extracellular proteins. J
25 Bacteriol 184, 3485-3491.

Schnappinger, D., Schubert, P., Pfeleiderer, K. and Hillen, W. (1998). Determinants of protein-protein recognition by four helix bundles: changing the dimerization specificity of Tet repressor. EMBO J 17, 535-543.

- Scholz, O., Thiel, A., Hillen, W., and Niederweis, M. (2000). Quantitative analysis of
30 gene expression with an improved green fluorescent protein. Eur J Biochem 267, 1565-1570.

- 5 Schreiber, V., Steegborn, C., Clausen, T., Boos, W., and Richet, E. (2000). A new mechanism for the control of a prokaryotic transcriptional regulator: antagonistic binding of positive and negative effectors. *Mol Microbiol* 35, 765-776.
- Schubert, P., Schnappinger, D., Pfeleiderer, K. and Hillen, W. (2001). Identification of a stability determinant on the edge of the Tet repressor four-helix bundle
10 dimerization motif. *Biochemistry* 40, 3257-3263.
- Schwebach, J. R., Chen, B., Glatman-Freedman, A., Casadevall, A., McKinney, J. D., Harb, J. L., McGuire, P. J., Barkley, W. E., Bloom, B. R., and Jacobs, W. R., Jr. (2002). Infection of mice with aerosolized *Mycobacterium tuberculosis*: use of a nose-only apparatus for delivery of low doses of inocula and design of an ultrasafe
15 facility. *Appl Environ Microbiol* 68, 4646-4649.
- Shimizu-Sato, S., Huq, E., Tepperman, J. M., and Quail, P. H. (2002). A light-switchable gene promoter system. *Nat Biotechnol* 20, 1041-1044.
- Smith, L. D. and Bertrand, K. P. (1998). Mutations in the Tn10 *tet* repressor that interfere with induction. Location of the tetracycline-binding domain. *J Mol Biol* 203,
20 949-959.
- Soukup, G. A., and Breaker, R. R. (1999). Engineering precision RNA molecular switches. *Proc Natl Acad Sci USA* 96, 3584-3589.
- Soukup, G. A., Emilsson, G. A., and Breaker, R. R. (2000). Altering molecular recognition of RNA aptamers by allosteric selection. *J Mol Biol* 298, 623-632.
- 25 Staudinger, B. J., Oberdoerster, M. A., Lewis, P. J., and Rosen, H. (2002). mRNA expression profiles for *Escherichia coli* ingested by normal and phagocyte oxidase-deficient human neutrophils. *J Clin Invest* 110, 1151-1163.
- Stormo, G. D. (2003). New tricks for an old dogma: riboswitches as *cis*-only regulatory systems. *Mol Cell* 11, 1419-1420.
- 30 Sudarsan, N., Barrick, J. E., and Breaker, R. R. (2003). Metabolite-binding RNA domains are present in the genes of eukaryotes. *RNA* 9, 644-647.

- 5 Suess, B., Hanson, S., Berens, C., Fink, B., Schroeder, R., and Hillen, W. (2003). Conditional gene expression by controlling translation with tetracycline-binding aptamers. *Nucleic Acids Res* 31, 1853-1858.
- Tang, J., and Breaker, R. R. (1997). Rational design of allosteric ribozymes. *Chem Biol* 4, 453-459.
- 10 Tuerk, C. and Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249, 505-510.
- Urlinger, S., Baron, U., Thellmann, M., Hasan, M. T., Bujard, H., and Hillen, W. (2000). Exploring the sequence space for tetracycline-dependent transcriptional
15 activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci USA* 97, 7963-7968.
- Uzzau, S., Figueroa-Bossi, N., Rubino, S., and Bossi, L. (2001). Epitope tagging of chromosomal genes in *Salmonella*. *Proc Natl Acad Sci USA* 98, 15264-15269.
- van Deursen, F. J., Shahi, S. K., CM, R. T., Hartmann, C., Guerra-Giraldez, C.,
20 Matthews, K. R., and Clayton, C. E. (2001). Characterisation of the growth and differentiation *in vivo* and *in vitro*-of bloodstream-form *Trypanosoma brucei* strain TREU 927. *Mol Biochem Parasitol* 112, 163-171.
- von Eggeling, F., Davies, H., Lomas, L., Fiedler, W., Junker, K., Claussen, U., and Ernst, G. (2000). Tissue-specific microdissection coupled with ProteinChip array
25 technologies: applications in cancer research. *Biotechniques* 29, 1066-1070.
- Weber, W., Fux, C., Daoud-El Baba, M., Keller, B., Weber, C. C., Kramer, B. P., Heinzen, C., Aubel, D., Bailey, J. E., and Fussenegger, M. (2002). Macrolide-based transgene control in mammalian cells and mice. *Nat Biotechnol* 20, 901-907.
- Weber, W., Schoenmakers, R., Spielmann, M., El-Baba, M. D., Folcher, M., Keller,
30 B., Weber, C. C., Link, N., van de Wetering, P., Heinzen, C., *et al.* (2003). Streptomyces-derived quorum-sensing systems engineered for adjustable transgene expression in mammalian cells and mice. *Nucleic Acids Res* 31, e71.

- 5 Weeks, K. M., Ampe, C., Schultz, S. C., Steitz, T. A., and Crothers, D. M. (1990). Fragments of the HIV-1 Tat protein specifically bind TAR RNA. *Science* 249, 1281-1285.
- Werstuck, G., and Green, M. R. (1998). Controlling gene expression in living cells through small molecule-RNA interactions. *Science* 282, 296-298.
- 10 Wilson, D. S., Keefe, A. D., Szostak, J. W. (2001). The use of mRNA display to select high-affinity protein-binding peptides. *Proc Natl Acad Sci USA* 98, 3750-3755.
- Winkler, W., Nahvi, A., and Breaker, R. R. (2002a). Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* 419, 952-956.
- 15 Winkler, W. C., Cohen-Chalamish, S., and Breaker, R. R. (2002b). An mRNA structure that controls gene expression by binding FMN. *Proc Natl Acad Sci USA* 99, 15908-15913.
- Winkler, W. C., Nahvi, A., Sudarsan, N., Barrick, J. E., and Breaker, R. R. (2003). An mRNA structure that controls gene expression by binding S-adenosylmethionine.
20 *Nat Struct Biol* 10, 10.
- Wissmann, A., Baumeister, R., Müller, G., Hecht, B., Helbl, V., Pfeleiderer, K. and Hillen, W. (1991). Amino acids determining operator binding specificity in the helix-turn-helix motif of *Tn10* Tet repressor. *EMBO J* 10, 4145-4152.
- Wray, L. V., Jr., Zalieckas, J. M., and Fisher, S. H. (2001). *Bacillus subtilis* glutamine
25 synthetase controls gene expression through a protein-protein interaction with transcription factor TnrA. *Cell* 107, 427-435.
- Yao, F., Svensjö, T., Winkler, T., Lu, M., Eriksson, C., and Eriksson, E. (1998). Tetracycline repressor, *tetR*, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum*
30 *Gene Ther* 9, 1939-1950.
- Zhang, L., Fan, F., Palmer, L. M., Lonetto, M. A., Petit, C., Voelker, L. L., St John, A., Bankosky, B., Rosenberg, M., and McDevitt, D. (2000). Regulated gene

- 5 expression in *Staphylococcus aureus* for identifying conditional lethal phenotypes and antibiotic mode of action. Gene 255, 297-305.

Zhang, Q., Harada, K., Cho, H. S., Frankel, A. D., and Wemmer, D. E. (2001). Structural characterization of the complex of the Rev response element RNA with a selected peptide. Chem Biol 8, 511-520.